The Potential for Breeding for Stemphylium Blight Resistance in the Genus *Lens*

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By

Rajib Podder

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ABSTRACT

Stemphylium blight (SB) caused by *Stemphylium botryosum* Wallr. is a widespread fungal disease and is becoming a serious threat of lentil in most of the lentil growing areas of the world including the temperate northern prairies of North America. It causes significant leaf damage with severe yield loss of lentil. The overall aim of this research was to identify sources of resistance to stemphylium blight and to determine the inheritance of resistance in recombinant inbred populations (RILs) for potential use in lentil breeding. Experiments were conducted to evaluate disease resistance of germplasm accessions selected from seven *Lens spp.* and in intraspecific and interspecific RIL population. Growth chamber, greenhouse and field trials in Saskatoon and Bangladesh were conducted. Seventy accessions selected from all wild species of the *Lens* genus were screened for SB resistance. An F_{7}-derived *Lens culinaris* intraspecific ('Eston' x PI 320937) RIL population (LR-39) with 96 lines along with checks was screened for SB resistance in the field at the University of Saskatchewan and at the field of Pulses Research Centre (PRC), Ishurdi, Bangladesh. An F_{7}- derived interspecific RIL population from the cross between resistant parents *Lens culinaris* cv. 'Eston' x *Lens ervoides* IG 72815, consisting of 123 lines was evaluated in greenhouse facilities at University of Saskatchewan using the highly aggressive *S. botryosum* isolate SB-19, and under field conditions of PRC, Ishurdi, Bangladesh.

Most of the *L. culinaris* accessions were susceptible to SB, whereas more than 70% of the wild lentil accessions had disease severity scores equal to or significantly lower than that of the SB resistant check 'Eston'. The highest frequency of resistance to SB was observed in *L. lamottei* followed by *L. ervoides* of the secondary gene pool. These sources can potentially be used to develop new commercial cultivars with multiple disease resistance. A number of lines from both RIL populations showed consistent disease reaction, either resistant or susceptible, in the two environments with a significant variation in each environment. A continuous frequency distribution indicated quantitative inheritance of resistance in both RILs with some transgressive segregates for resistant disease reaction in the Bangladesh environment for LR-39, and for LR-26 compared to the resistant *L. culinaris* parent 'Eston' under Saskatoon and Bangladesh field conditions, respectively. Fifty-seven or 33% of RILs from the intraspecific LR-39 population showed resistance to SB in

comparison to ‘Eston’. For the interspecific population LR-26, 60% and 68% of RILs had either resistant or very resistant disease reactions, respectively, under natural inoculation pressure in the field in Bangladesh and under greenhouse conditions when inoculated with isolate SB-19 at Saskatoon. The genotype × location interaction variance was higher than the genotype variance, indicating a substantial influence of the environment on the expression of resistance. Selected resistant RILs from both environments can be given priority for incorporation in the breeding program, and gene pyramiding might be helpful to develop new cultivars with resistance to multiple diseases including SB in the future. A high proportion of interspecific breeding lines with consistent resistant reaction to SB should prove useful for future breeding strategies. This extensive study of SB resistance inheritance pattern will provide a base for identification of potential SB resistance genes or interspecific origin through marker assisted breeding.

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DEDICATION

To all of my well wishers

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List of abbreviations used

BARI	Bangladesh Agricultural Research Institute
CDC	Crop Development Centre
DAS	Days after sowing
DS	Disease severity
L.	*Lens*
PE	Polyethylene
PRC	Pulses Research Centre
RCBD	Randomized Complete Block Design
RIL	Recombinant Inbred Line
S.	*Stemphylium*
SB	Stemphylium blight
SB 19	*Stemphylium botryosum* isolate 19
U of S	University of Saskatchewan

CHAPTER I

1. INTRODUCTION

Pulse crops have been traditionally used mainly in human diets with cereals around the world. Among the pulses, lentil (*Lens culinaris* Medik. ssp. *culinaris*) is a diploid (2n=2x=14 chromosomes) that is believed to have been domesticated and consumed since pre-historic times (Yadav et al., 2007). World production of lentil in 2010 was estimated at 4.58 Mt from an estimated 4.18 million ha with an average yield of 1.09 t ha^{-1} (Food and Agriculture Organization of the United Nations, 2010). Canada produced 1.95 Mt of lentil from 1.34 million ha in 2009, an average yield of 1.46 t ha^{-1} (Food and Agriculture Organization of the United Nations, 2010). Production in Canada increased to more than 1.40 million ha in 2010 and then slightly decreased in 2011 to 1.04 million ha (Statistics Canada, 2012).

Lentils are an important source of dietary protein and when used in combination with cereals they provide adequate amounts of balanced essential amino acids in human diets and animal feed. The chemical composition of raw lentils revealed an appreciable amount of protein, lipid and carbohydrate at 20.6, 2.2 and 56.4 g/100g, respectively (Costa et al., 2006). The lentil crop is also an important component of legume crop diversification in the predominantly cereal-based cropping systems in the Gangetic plain of South Asia and in Saskatchewan. Moreover, lentil crops can improve soil nutrient status through symbiotic nitrogen fixation, conserving soil moisture and limiting soil erosion (Muehlbauer et al., 1992).

Lentil plants are affected by different biotic and abiotic constraints that limit yield and seed quality. Fungal diseases are major factors for yield instability of lentil throughout its geographical distribution. Some of them are common in all lentil growing areas and some diseases occur in specific areas. Ascochyta blight (caused by *Ascochyta lentis* Vassilievsky , anthracnose (*Colletotrichum truncatum* (Schwein.) Andrus and Moore), fusarium wilt (caused by *Fusarium oxysporum* Schlecht: Fr. f. sp. *lentis* Vasudeva and Srinivasan), botrytis (caused by *Botrytis cinera* Pers.: Fr.)., sclerotinia white mold (caused by *Sclerotinia sclerotiorum* (Lib.) de Bary) and stemphylium blight (caused by *Stemphylium botryosum* Wallr.) are the most common fungal

diseases of lentil in the temperate northern prairies of North America and in the Gangetic plain of south Asia (Saha, 2009; Kumar, 2007).

Stemphylium botryosum causes leaf blight on lentil that can result in large-scale defoliation of plants. Stemphylium blight (SB) is considered as a serious disease for the lentil growers as well as lentil researchers in Bangladesh. Lentil yield can be reduced by 88% due to SB and its distribution is almost uniform throughout the country where changing of climatic factors is considered as a major factor for increasing its severity rate year after year. Once thought of as a minor disease of lentil with local significance in south Asia (Sharma, 2009), it is now considered important in many of the world's major production regions. It is now widespread and has been reported in lentil production areas of Bangladesh, India, Nepal, the USA, Syria, Egypt and Canada (Erskine and Sarker, 1997; Morrall et al., 2004; Bayaa, and Erskine, 1998). In recent years SB has been observed increasingly in lentil fields in Saskatchewan and on seed tested for infection with *A. lentis* and other common lentil pathogens (Banniza et al., 2005). Limited research is underway to develop SB resistant varieties of lentil at various research institutions such as the University of Saskatchewan, the Bangladesh Agricultural Research Institute (BARI), the Indian Agricultural Research Institute (IARI) in India and the National Agricultural Research Centre (NARC) in Nepal. A comprehensive breeding strategy for SB would be useful for improvement of lentil varieties in many parts of the world.

Lentil is now grown in diversified environments around the world. Tullu et al. (2010) described three main climatic regions for current lentil production, each accounting for roughly a third of current global supply. These are (1) the winter production regions of the Mediterranean (where lentil evolved) and Southeastern Australia, (2) the sub-tropical winter production region of South Asia where lentil was introduced from the centre of diversity and (3) the summer temperate regions encompassing the northern grain belt of the North American prairies. In these three distinct zones lentil crops experience different environments to complete their life cycle. Direct introduction of genotypes from one to the other is virtually impossible because of poor photoperiod-temperature adaptation (Tullu et al., 2010). Natural selection, extensive artificial selection pressure and breeding programs for genotypic improvement of lentil that narrow the genetic base may increase susceptibility to

different biotic stresses. In this case interspecies crosses between different species from the *Lens* genus or introgression of genes from the wild gene pool might be useful to gather variability for future use. There is evidence for resistance sources for different diseases like ascochyta blight, rust, drought and for moisture stress in the wild gene pool (Tullu et al., 2010; Gupta and Sharma, 2006; Bayaa et al., 1994). Recent research in Canada led to the development of anthracnose resistant varieties. After screening a large number of accessions from both the subspecies of *Lens culinaris* (ssp. c*ulinaris* and *orientalis*), only a few accessions had some level of resistance to race 1 of anthracnose (Buchwaldt et al., 2004; Tullu et al., 2006). No accessions were found with resistance to the more aggressive race 0, but in the secondary and tertiary gene pool, a high frequency of accessions resistant to both anthracnose races was observed (Tullu et al., 2006).

The development of disease resistance for genetic improvement is a continuous process because released varieties become susceptible over time due to environmental change and evolution of new pathotypes. As resistance to major pathogens is deployed, minor diseases or new diseases may develop. For example, prior to the widespread use of lentil varieties with resistance to ascochyta blight, SB was not considered a major disease in Saskatchewan, but now it is becoming a threat for lentil growers. Identification and development of sources of resistance to SB is an important objective of the lentil breeding program. It may be possible to find sources of resistance to SB from the secondary and tertiary gene pool of lentil. Tullu et al. (2010) also reported some preliminary evidence that there might have a high level of resistance to SB in lines derived from hybrids between cultivated lentil and *Lens ervoides.*

To date, there have only been a few published investigations into the genetics of SB resistance and management of SB in lentil. This disease has become a more serious problem with the increase of lentil production and deployment of resistance to ascochyta blight and anthracnose in new cultivars (Vandenberg and Morrall, 2002). Research studies have been initiated to develop appropriate field and indoor techniques for phenotyping SB reactions in lentil with the objective to study the inheritance of resistance in lentil (Saha, 2009; Kumar, 2007; Banniza and Vandenberg, 2009). Germplasm from the secondary and tertiary gene pools and some

parents of recombinant inbred line (RIL) populations can be evaluated to identify sources of genetic resistance to SB. The results of such studies may play an integral role in developing a long term strategy for resistance breeding to SB.

1.1 Research hypotheses and objectives

Research is underway at the Crop Development Centre to develop lentil cultivars with genetic resistance to SB as part of a long-term strategy for reducing potential losses caused by the disease. The long term goal of this research initiative is to contribute scientific knowledge that will help in the development of a sound breeding strategy for SB resistance in lentil for western Canada. This could lead to effective screening methodologies, genetic characterization of SB resistance and use of marker assisted selection techniques for breeding. Considering these issues the hypotheses for this specific research project were:

- Cultivated and wild lentil germplasm collections have accessions with resistance to stemphylium blight
- Resistance to stemphylium blight is transferable from *L. culinaris* resistant sources to the cultivated species
- Resistance to stemphylium blight is transferable from wild species to the cultivated species

The specific objectives of this project were:

- Identification of new sources of resistance to SB in the cultivated lentil and in the wild species of *Lens.*
- Estimation of inheritance for SB resistance under indoor and field conditions with local germplasm
- Development of a robust SB resistance breeding strategy based on integration of new knowledge in pathology and genetics

As part of this project, Canadian and foreign lentil germplasm, parents of existing RIL populations segregating for SB resistance and wild relatives of *Lens culinaris* were used as important potential genetic sources for developing genetic resistance to the disease.

The experiments described in the thesis are presented in manuscript format as Chapters 3, 4 and 5. Chapter 3 was accepted for publication in Plant Genetic Resources: Characterization and Utilization.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Derivation, domestication and distribution of lentil

Lentil (*Lens culinaris* Medik.) is considered one of the oldest domesticated plant species among the primary crops that originated from the Near Eastern complex, like einkorn, emmer, barley, linseed and pea (Harlan, 1992). The earliest evidence of lentil as carbonized remains was reported from Greece's Franchthi cave dated to 11,000 BC (Sandhu and Shing, 2007). The wild ancestor of cultivated lentil (subsp. *orientalis*) was found throughout the Fertile Crescent (Pearman, 2005). Lentil is an annual herb with slender stems, branching canopy structure and light green, pubescent foliage (Duke, 1981). Archeological data representing the distribution of wild species revealed the Near East as a valuable source for both the wild species and cultivated species of *Lens* (Cubero et al., 2009), but lentil is now well adapted and grown in temperate and semi-arid regions on all continents (Muehlbauer et al., 2009).

Commercial production of lentil in Western Canada began in 1970, when approximately 600 ha were grown. Now lentil is a very important crop of western Canada due to its ability to improve economic returns to growers, its value in crop diversification and due to its reduced requirement for nitrogen fertilizer (Saskatchewan Agriculture Statistics, 2012). Production has increased in Saskatchewan to as much as 1.04 million ha in 2011 (Statistics Canada, 2012).

There is remarkable diversity in consumption of lentil on the basis of culinary tradition, cotyledon colour and value addition. Lentil is mainly consumed in South Asia, West Asia, North Africa, Mediterranean countries, Egypt and Turkey as a dhal (Sarker and Erskine, 2006; Muehlbauer et al., 2009). The three nations Canada, the USA and Australia, which are low per capita consumers of lentil, produced more than 50% (2.47 Mt out of 4.58 Mt) of the total world lentil produced in 2010 (FAOSTAT, 2010). Both green and red lentils are grown in Saskatchewan with six market classes.

2.2 Taxonomy and classification of lentil

Lentil belongs to the genus *Lens* of the family Leguminosae. Tournefort first used the word "Lens" to designate a specific genus followed by Miller and Adanson (reviewed

in Cubero et al., 2009). Association was found between the genus *Lens* and genera of the tribe *Vicieae* (Zohary et al., 2012). Barulina (1930) classified cultivated lentil into two sub-species with an ample collection from the diverse locations of the world, mainly on the basis of seed size. The subspecies *macrosperma* consists of large seeded, yellow cotyledon lentil with a seed size that range from 6 to 9 mm diameter, whereas the subspecies *microsperma* are lentil with small seeds with red, orange or yellow cotyledons and 2-6 mm diameter seed size (Barulina, 1930; Bermejo et al., 2010). An intensive morphological study of the *Lens* genus by principal component analysis (PCA) revealed that *L. culinaris* is closest related to *L. orientalis* followed by *L. nigricans*, whereas *L. ervoides* is more unique than others (Hoffman et al., 1988). Cubero et al. (2009) reviewed different studies conducted since 2001 for the genetic grouping of *Lens* species based on several criteria. Tullu et al. (2010) analyzed the phylogeny of *Lens* on the basis of Cubero's review of hybridization barriers. In this scenario, the *Lens* genus has *L. culinaris* (with ssp. *culinaris* and *orientalis*) in the primary gene pool, *L. odemensis* and *L. tomentosus* in the secondary gene pool and the remaining three species (*L. nigricans, L. ervoides* and *L. lamottei*) are in either the secondary or tertiary gene pools.

2.3 Diseases of lentil

One of the major limitations to improvement of lentil yield worldwide is several infectious diseases, especially plant pathogenic fungi. For instance, ascochyta blight (caused by *Ascochyta lentis* Vassilievsky) and fusarium wilt (caused by *Fusarium oxysporum* Schlecht: Fr. f. sp. *lentis* Vasudeva and Srinivasan) are considered to be worldwide (Chen and Sharma, 2011). Other foliar disease such as rust (caused by *Uromyces vicia-fabae* (Pers.) J. Schroet.), stemphylium blight (caused by *Stemphylium botryosum* Wallr.), sclerotinia white mold (*Sclerotinia sclerotiorum* (Lib.) de Bary) and grey mould (caused by *Botrytis cinerea* Pers.: Fr.) are also reported as major diseases and prevalent in certain areas (Saha, 2009; Chen and Sharma, 2011). Chongo et al. (2002) reported anthracnose caused by *Colletotrichum truncatum* (Schwein.) Andrus and Moore, botrytis grey mould and sclerotinia white mould are major problems in North America. Collar rot caused by *Sclerotium rolfsii* Sacc. is also found in certain areas with high humid conditions. Powdery mildew (caused by *Erysiphe trifolii*) has also been reported to affect lentil in the Pacific

Northwest (PNW) of the United States and Canada (Attanayake et al., 2009; Banniza et al., 2004).

Stemphylium blight is a major problem in Bangladesh, northeast India and Nepal and in recent years, it has appeared in Saskatchewan and North Dakota (Holzgang and Pearse, 2001; Kumar, 2007). Seedling blight and root rot caused by *Rhioctonia solani* Kühn, *Aphanomyces euteiches* C. Drechsler, and *Pythium ultimum* Trow., are also important threats to lentil production.

The viral diseases are the second most important group of lentil pathogens and have appeared in different lentil growing areas of the world. Six viruses have been reported cause yellowing, stunting/necrosis and ten viruses for mosaic or mottling symptoms (Kumari et al., 2009). Among the viruses that infect lentil, bean leaf roll virus, beet western yellow virus, bean yellow mosaic virus, broad bean stain virus, and pea seed borne mosaic virus are the most widely distributed. Recently, pea enation mosaic virus was observed in exotic materials in Bangladesh (Boss et al., 1988; Saha, 2009).

Three different types of broomrape, an obligate root parasitic angiosperm weed, are reported as a serious threat for lentil cultivation in Mediterranean region (Fernandez-Aparicio et al., 2009). *Orobanche crenata* is the most common lentil parasite in the Mediterranean Basin, Middle East, Andalusia and southern Spain.

2.4 Genetics of disease resistance

The study of genetics of plant disease resistance to different pathogens began in the 19th century with the aim of identifying resistance genes and introgression of those into improved cultivars (Saha, 2009). Host resistance can be expressed in various forms and regulated by numerous genetic systems. Inheritance of disease resistance is either qualitative or quantitative. Hooker and Saxena (1971) classified it into oligogenic, polygenic and extra-chromosomal categories. The gene-for-gene theory (Flor, 1947) explained qualitative resistance where the resistance gene of the host and the avirulence gene of the pathogen are believed to express the phenotype following Mendelian theory. Inheritance and regulation of quantitative resistance is still poorly understood. The concept of disease resistance can be quite complex but a clear

understanding can provide opportunities for use in crop improvement programs. The results of a study by Tar'an et al. (2003) showed that pyramiding of genes for resistance to ascochyta blight and for resistance to anthracnose can be used in lentil breeding, A recombinant inbred line (RIL) population ($F_{6:7}$) consisting of 156 lines was developed from a cross between 'CDC Robin' and a breeding line '964a-46'. Eleven RILs retained all the three resistance genes. If SB resistance genes can be identified, it might be possible to develop multiple resistance cultivars by including SB resistance gene in the gene pyramiding procedure.

2.5 *Stemphylium spp*. cause Stemphylium blight – a case study with lentil

2.5.1 Taxonomy and morphology of the pathogen

The genus *Stemphylium* belongs to the family *Pleosporacea* in the order *Pleosporales*, believed to have selfing species that evolve from outcrossing ancestors (Inderbitzin et al., 2005). *Stemphylium botryosum* Wallr. is the causal organism of SB of lentil, an asexual stage, whereas *Pleospora herbarum* is its sexual stage, the telomorph of *Stemphylium* (Inderbitzin et al., 2009, Bayaa and Erskine, 1998). The genus *Stemphylium* has both saprophytic and pathogenic species and many of them show pathogenicity on a wide range of economically important crops (Farr et al., 1989). Identification of different species of *Stemphylium* by observing the morphology of conidia, conidiophores and ascospore morphology is quite complicated, but can be improved by molecular characterization (Camara et al., 2002). Wang et al. (2010) also supported this result while studying two new species of *Stemphylium* (*Stemphylium phaseolina* and *S. variabilis*), isolated from diseased leaves of *Phaseolus vulgaris* L. in Hebei Province, China, and from diseased leaves of *Allium sativum* L. in Anges, France, respectively. They found distinctness between these two species on the basis of morphological characters and molecular phylogenetic analyses.

Stemphylium spp. can be identified by their conidia with both transverse and longitudinal septa, with ends rounded, symmetrical from end to end. *Stemphylium. botryosum* Wallr., produces airborne conidia developed from short, aseptate conidiophores occurring singly or in groups and characteristically, *Stemphylium botryosum* conidia are slightly constricted in the middle (Pitt and Hocking, 2009; Bayaa and Erskine, 1998).

2.5.2 Prevalence and development of stemphylium blight disease

Stemphylium blight is one of the most devastating diseases of lentil in Bangladesh, Nepal and north-eastern India (Chen et al., 2009; Bakr and Ahmed, 1992). It has also been reported on lentil in Canada, Syria, Egypt, Hungary and the USA (Bayaa and Erskine, 1998; Morrall et al., 2006; Chen et al., 2009). Results from a laboratory test with many samples from central and northern districts of Saskatchewan revealed high levels of *Stemphylium* spp. (Morrall et al., 2006). In 2008, it was observed in less than a quarter of lentil crops in Saskatchewan (Barker, 2009). It can cause yield losses above 80% in Bangladesh, eastern Nepal and north-eastern India (Bakr and Ahmed, 1992). Vandenberg and Morrall (2002) reported that SB had become a more serious problem with the increase of lentil production and cultivation of new cultivars with resistance to ascochyta blight and anthracnose. In recent years, *Stemphylium* has been observed with increasing frequency in lentil fields in Saskatchewan when seed tested for infection with *A. lentis* and other common lentil pathogens (Banniza et al., 2005).

The biology of *S. botryosum* makes it a potential threat for lentil cultivation. The fungus can produce airborne conidia and can germinate with multiple germ tubes that help to penetrate the host, primarily through stomata, followed by invasion into the plant tissues including seeds (Chen et al., 2009). Infected seed, dead stems, and leaves can provide inoculum for infection in succeeding years (Saha, 2009). Survival of pseudothecia of *Pleospora herbarium* through over-wintering in plant debris for a long period was reported in garlic (*Allium sativum)* (Basallote- Ureba et al., 1999) and in alfalfa (*Medicago sativa*) (Elmer et al., 1996). Solfrizzo et al., (1994) reported phytotoxin stemphol produced from some isolates of *S. botryosum* that has been implicated in lesion formation on various hosts. A significant positive relationship was observed between phototoxic substances produced by *S. botryosum* f.sp. *lactucum* with disease symptoms developed on susceptible lettuce (*Lactuca sativa*) cultivars (Barash et al., 1978). Though seed infection by *S. botryosum* has been reported in previous studies, there is no clear understanding of the significance of seed borne *S. botryosum* inoculum on disease initiation of lentil (Mwakutuya, 2006).

2.5.3 Lentil and Other Hosts affected by *Stemphylium* spp.

Significant levels of diversity among *Stemphylium* spp. has helped to cause disease in several hosts and the loss due to different species are also diversified in different regions. Sometimes the same species infects different hosts. *Stemphylium botryosum* of lentil was first identified in 1987 in Bangladesh (Bakr and Zahid, 1987). Different unpublished reports suggest that it can cause up to 83-90% yield loss in different areas of India. Complete crop damage due to SB infection was also reported in Bangladesh in recent years (Sarker et al., 2004). As the cultivated area of lentil increases in western Canada, SB may become an increasing threat to lentil production, particularly if higher levels of resistance are achieved and maintained for other major foliar diseases like ascochyta blight and anthracnose (Hashemi et al., 2005a).

Stemphylium blight was reported in onion (*Allium cepa)* in Spain (Maria et al., 1998) and for the first time in Egypt by *Stemphylium vesicarium* (Hassan et al., 2007). Leaf blight of garlic is a destructive disease caused by *Stemphylium solani* in Hubei province, China. Symptoms were observed on infected leaves in Dangyang County from autumn 2004 to spring 2007, with the diseased area estimated to be over 7000 ha. Garlic yield was reduced by 30% on average with up to 70% yield loss in some fields (Zheng et al., 2007). The occurrence of stemphylium leaf spot on asparagus (*Asparagus officinalis*) caused by both *Stemphylium vesicarium* and *S. botryosum* was reported in Victoria, Australia by Cunnington and Irvine (2005). An epidemic caused by *S. solani* on cotton (*Gossypium hirsutum*) was reported from Parana state of Brazil during 1994 and 1995, and caused up to 100% yield losses in a local susceptible cotton cultivar in India (Mehta, 1998). Severe leaf blight of tomato caused by *Stemphylium botryosum* was recently reported in England (Dickens and Evans, 2007). The pathogenicity of the fungus was confirmed, and this is believed to be the first record of this fungus attacking tomato foliage in the UK. Berg and Leath (1996) reported stemphylium leaf spot, caused by *S. sarciniforme* on red clover (*Trifolium* spp.) at high severity levels in the northeastern USA and Canada during moist weather condition. A newly identified fruit rot disease of sweet pepper (*Capsicum annum*) was reported in Japan at 2011, caused by *S. lycopersici* (Tomioka and Sato, 2011). In China, three new species of *Stemphylium* were recently identified by both morphological observations and molecular study in *Luffa cylindrica*, *Lycium*

chinense and *Cucumis melo* and named as *Stemphylium luffae, S. lycii* and *S. cucumis*, respectively (Pei et al., 2011).

2.5.4 Symptoms of Stemphylium blight

The development of SB on lentil first appears on lentil leaflets as small, light beige lesions. While the disease is most readily apparent when blighted leaves are noticed at the top of the canopy, it is likely present under the canopy as well. Eventually, smaller lesions merge to produce larger, irregularly shaped lesions that can kill entire leaflets and branches (Barker, 2009). Prolonged moist periods promote further infections and give the upper canopy a grey-brown appearance. Infected leaflets may fall to the ground, and serve as a source of spores for future infections of a wide range of plants. Fungal spores can be produced on older lesions and appear dark brown and fuzzy. Leaves may twist and roll due to desiccation caused by the pathogen. Stemphylium blight usually affects different crops from the flowering stage onwards. Infection can occur on all the aerial parts of lentil plants, such as leaflets, pedicels, flowers, and entire branches and results in a blighted appearance (Chen et al., 2009). They also reported significant reduction of plant biomass, lower seed yield, decreased seed size, seed staining and lower germination rates from severely infected fields. A number of crops that are affected by SB show more or less similar symptoms. For example, onion plants exhibited symptoms of blight on the leaves and seed-stalk and initial symptoms on leaves consisted of tip necrosis followed by small white and/or large purple spots (Hassan et al., 2007).

2.5.5 Studies of the genetics of SB disease resistance

Sharma (2009) reported SB as a locally important disease in South Asia, where resistance sources are also available. Genetics and inheritance of resistance to stemphylium blight of lentil have not been completely elucidated (Saha, 2009). Kumar (2007) studied a recombinant inbred line (RIL) population of lentil, developed from the cross BARIMasur-4 X CDC Milestone. Based on the frequency distribution it was concluded that resistance to SB was quantitatively inherited. In a study to determine the inheritance and linkage map positions of genes conferring resistance to SB in lentil, Saha et al., (2010) found a complex inheritance of SB after screening 206 F_7-derived lines developed by crossing between *L. culinaris* parents ILL 6002

(resistant) and ILL 5888 (susceptible) at PRC, Ishurdi, Bangladesh. In two different years with the same population, they reported one and three significant quantitative trait loci (QTL) based on disease scores from 2006-2007 and 2008-2009 experiments. One QTL (QLG4 $_{80\text{-}81}$) was common in both years.

2.5.6 Environmental factors that influence stemphylium blight

Basically temperature, host and environment interaction influence disease development. Understanding the role of the environment on the development of SB in lentil is an important area to control the disease effectively. Lentil grows in different agro-climatic regions, for instance, the total life cycle of lentil is completed in the winter in South Asia, whereas in western Canada, cold winters are followed by a short summer. The places where SB now occurs may be due to the survival capacity of the pathogen and pathotype diversity in different environments. Saha (2009) reported that most of the research on infection by *Stemphylium* spp. of different hosts has confirmed that temperature and moisture are the two most important environmental factors. A wide range of temperature (5 to >30°C) was reported for conidial germination and infection of lentil in the presence of free water (Chen et al., 2009). The authors also reported that saprophytic growth of *S. botryosum*, infected lentil debris from previous years and a wide range of alternative hosts help the pathogen to survive and provide inoculum for the next crop. A significant positive relationship was observed between leaf wetness period up to 24h and increase of infection rate for the necrotrophic fungus *Didymella rabiei* on chickpea (Jhorar et al., (1998). Mwakutuya (2006) explored in detail optimum temperature and humidity (%) for higher SB severity in controlled conditions after inoculation with *S. botryosum*. Sinha and Singh (1993) reported that high relative humidity, cloudy days and moderate to warm temperatures are the optimal conditions for SB epidemics in lentil in Indian tropical or sub-tropical environment. The effect of light and temperature on sporulation of *Stemphylium botryosum* f. sp. *lycopersici* was also reported on tomato plants in *in vivo* conditions (Bashi and Rotem, 1975). The highest sporulation was found at 25°C with 24 continuous darkness and wet period. A direct and positive relationship was reported between the concentration of *S. vesicarium* spores in the air and both the amount of rainfall and number of hours with a temperature range of 12-21°C (Prados-Ligero et al., 2003).

2.5.7 Strategies for control of stemphylium blight

At this time, no fungicides are registered for control of stemphylium blight on lentil in Canada (Barker, 2009). The effect of fungicides on colony growth of *S. botryosum* showed that the inhibition of growth was influenced by the doses and the fungal pathogen. Lance was most effective against *S. botryosum*, while Headline ® EC 250 g/L was intermediate (Banniza, et al., 2005). They also reported that Lance® and Quadris® X-Factor ™ reduced SB significantly compared to the control on inoculated lentil plants. Benomyl (Benlate 50WP, DuPont Canada Inc., Mississauga, ON) and propiconazol (Tilt 250 EC, Syngenta Crop Protection, Inc. Greensboro, NC.) were found effective to reduce SB (by *S. botryosum*) disease incidence in an alfalfa field trial (Hwang et al., 2006). In Bangladesh and India, SB of lentil is managed through late seeding, fungicide applications and the use of resistant cultivars. Bakr and Ahmed, (1992) reported that after observing the first symptoms, three applications of iprodione, propineb, mancozeb or sulfur, can be sprayed at seven day intervals that can significantly reduce the severity and increase yield by 23-40%. One bacterial strain of *Streptomyces sp.* CIMAP-A_1 was found (in vitro) to have antagonistic effects on *Stemphylium spp.* (Alam et al., 2012) hence can be used as a bio-fungicide to control seed borne *Stemphylium* as suggested by Marja-Leena (2003).

2.5.8 Resistant germplasm sources to stemphylium blight

There are known sources of genetic resistance to SB of lentil. The International Centre for Agricultural Research in the Dry Areas (ICARDA) has approximately 10,000 accessions of *Lens culinaris* collected from 86 countries of the world, the largest global germplasm resource for national lentil research programs (Sarker et al., 2005). Three significantly resistant cultivars (BARIMasur-4, BARIMasur-5 and BARIMasur-6) were developed at the Pulses Research Centre (PRC), Bangladesh in cooperation between ICARDA and BARI. After evaluating different lentil cultivars in growth chambers at the University of Saskatchewan for resistance to stemphylium blight, Banniza et al., (2005) reported that BARIMasur-4 and 'Eston' were the most resistant cultivars with significantly lower disease levels compared to all other lentil cultivars. CDC Glamis was the most susceptible cultivar with significantly higher levels of stemphylium blight compared to all other lentil cultivars tested. CDC Blaze, CDC Milestone, CDC Redcap, CDC Robin and CDC Vantage had comparable,

intermediate levels of resistance. Another field screening at the Crop Development Centre (CDC) of the University of Saskatchewan with some *L. culinaris* cultivars showed inconsistent disease severity compared to results of previous screening under controlled conditions of the same cultivars (Banniza & Vandenberg 2009).

2.5.9 Disease ratings and assessments

Selection or development of rating scales for different diseases depend on the nature of a particular disease, pathogen biology and host pathogen interaction. Various methods have been developed for SB screening and assessments by different researchers. Chen (2007) used a 0-9 scale to phenotype SB disease reaction, taking into account leaf area infection during the late flowering stage. This scale was also used by Saha, (2009) to screen a RIL population in field conditions. Banniza et al., (2005) reported that the Horsfall-Barratt scale was used to screen stemphylium blight. Difficulties in the statistical analysis using the Horsfall-Barratt scales guided them to develop a new semi-quantitative scale. This new scale (Table 2.1) is relatively simple and considers lesion development with leaf drop, allowing evaluation of large numbers of plants in less time. Kumar, (2007) used this scale for screening lentil cultivars and RILs for SB.

Table 2.1. Scaling techniques for assessing stemphylium blight of lentil based on appearance of chlorotic symptoms and gradual leaf drop

Scale	Symptom
0	healthy plants
1	few tiny lesions
2	a few chlorotic lesions expanding lesions on leaves, onset of leaf drop
3	1/5th of nodes affected by lesions and leaf drop
4	2/5th of nodes affected
5	3/5th of nodes affected
6	4/5th of nodes affected
7	All leaves dried up
8	All leaves dried up but
9	Stem green
10	Plant completely dead
Sources:	Hashemi et al., 2005

Recently a field screening was done using a 0-10 scale for assessing the severity of stemphylium blight in lentil (Banniza and Vandenberg, 2009), where 0 = 0% DS, 1 = 1-10% DS, etc. and 10 = 91-100% DS).

2.6 Wild species as a potential source for resistance to plant pathogen

Wild species of different crops have been using as a potential source of resistance for biotic and abiotic stresses due to their wide genetic diversity. A narrow genetic base and a higher sensitivity to unfavorable biotic and abiotic stresses of different cultivated crop species have led to searches for resistance sources from different wild relatives. For instance, lack of variability among the indigenous lentil was considered a bottleneck for plant breeding progress in South Asia, the largest lentil growing area in the world (Erskine et al., 1998).

To date, resistance sources for ascochyta blight, anthracnose, rust, vascular wilt, cold and drought have been identified in different species of the *Lens* genus (Philip et al., 2007). ICARDA has more than 500 accessions of different wild species of *Lens* (Sarker et al., 2005; Tullu et al., 2010). The CDC at University of Saskatchewan obtained a large number of wild accessions from ICARDA. These were phenotyped under both field and greenhouse conditions by Tullu et al. (2006, 2010) for anthracnose and ascochyta blight, respectively. Significant numbers of resistant accessions were identified from *L. ervoides, L. nigricans, L. tomentosus* and *L. culinaris* ssp. *orientalis*. Though no studies have reported resistance source for SB from wild species, Tullu et al. (2010) did report some preliminary evidence that there might be a high level of resistance to SB in lines derived from hybrids between cultivated lentil and *Lens ervoides* accession L01-827A. Kim et al. (2008) reported direct or indirect Avr-R protein interaction between resistant wheat lines and the necrotrophic *Mycosphaerella graminicola* fungus. Resistance sources for race 0 and race 5 for *Fusarium oxysporum* f.sp. *ciceris* was reported in accessions of the wild chickpea species *Cicer bijugum* K.H. Rech. and *C. judaicum* (Boiss.) which belong to the tertiary gene pool (Kaiser et al., 1994). The former species was also reported to be completely resistant to *Ascochyta rabiei* (Pass.) Lab.) (Collard et al., 2001).

2.7 Screening protocol for Stemphylium blight resistance

2.7.1 Variation between isolates of *Stemphylium botryosum*

Kumar (2007) used four isolates (SB-16, SB-17, SB-19 and SB-BAN) in the Pulse Crop Pathology laboratory of the University of Saskatchewan to identify the most

suitable isolate for screening in indoor condition and found significant differences in conidia production. Isolates SB-19 and SB-BAN produced more conidia than the other two. Saha et al., (2010) used *Stemphylium blight* isolates I_1, I_2 and I_3 collected from the USA. A study carried out in the Plant Pathology laboratory of the Bangladesh Agricultural Research Institute (BARI) identified cultural and physiological variation among four *S. botryosum* isolates (MIH-1, MIH-2, MIH-3 and MIH-4) from different regions of Bangladesh. (Hosen et al., 2009). Marked variation was observed for colony color, shape, texture and conidial size.

2.7.2 Media for sporulation

Different media were screened and incubated for 11 days in light at 27°C to identify an appropriate media for sporulation (Banniza et al., 2005). Comparison of sporulation on various media revealed that among media tested with *S. botrysum* isolate SB-19, highest sporulation was obtained on V8 juice media with 43 to 55 x 10^4 spores mL^{-1} followed by lentil powder agar (LPA) at a concentration of 50% with 16 to 42 x 10^4 spores mL^{-1} and 25% potato dextrose agar (PDA) with 53 to 76 x 10^4 spores m/L. An additional experiment revealed that addition of PDA to the V8 juice medium dramatically increased sporulation by approximately 300 % compared to straight V8 juice medium (Banniza, et al., 2005). For large scale inoculation in controlled conditions, preparation of spore suspensions is not highly efficient. A mycelial suspension system for SB disease screening was found to be as efficient as spore suspensions (Hashemi et al., 2005b). Saha et al., (2009) also used PDA and V8 for sporulation of conidia followed by transfer of mycelium to potato (*Solanum tuberosum* L.) dextrose broth for 10 days and his work was similar to Chowdhury et al., (1996).

2.7.3 Optimum incubation condition for sporulation and germination

A preliminary experiment was conducted in Pulse Crop Pathology laboratory of the University of Saskatchewan to identify the optimum incubation period suggesting that

25 day incubation period in the light may be the most efficient approach for producing spores followed by 20 hours for spore germination (Banniza et al., 2005).

2.7.4 Concentration of spore for inoculation

Usually higher concentration (e.g. $1x10^5$ spores mL^{-1}, $2X10^5$ spores mL^{-1}) was found to better estimate disease severity. But for breeding purposes, Banniza et al., (2005) suggested that intermediate disease severity is desirable to identify subtle differences between genotypes, since SB is considered to be a quantitative trait. So, $2x10^4$ spores mL^{-1} or $1x10^5$ spores mL^{-1} was used for inoculation.

2.7.5 Inoculation of plants

Two drops of Tween® 20 with conidial suspension facilitated conidium-plant tissue contact by reducing the surface tension of water (Kumar, 2007). Inoculum was applied at four different growth stages, 14 DAP, 28 DAP, 42 DAP and 56 DAP, using an air-brush to evenly apply the conidial suspension followed by incubating for 48 hours in a mist chamber at 95% humidity and 20° C following inoculation. Saha et al. (2010) sprayed mycelial suspensions for disease screening. This method was considered as reliable as the spore suspension method.

2.7.6 Appropriate plant age for inoculation

Plant age is an important factor for artificial inoculation to obtain accurate and relevant data for scoring disease symptoms. A significant increasing trend of disease severity for SB was observed with increasing age of lentil plants (Banniza et al. 2005). Consequently, for breeding purposes, two to four weeks old plants were preferred for *S. botryosum* inoculation to differentiate between susceptible and resistance genotypes. Saha et al. (2009) inoculated *Stemphylium botryosum* isolate at eight weeks of plants age in his indoor screening.

2.8 Prologue to Chapter 3

To fulfill the objectives of this part of the research program, a number of experiments were planned for growth chambers and greenhouses at the University of Saskatchewan, and under field conditions at Saskatoon and PRC, Ishurdi, Bangladesh.

Results from all the experiments are grouped into three chapters. Chapter 3 presents the results from screening non-random selections of *L. culinaris* genotypes for SB resistance in four different environments. Results are also reported for SB resistance screening of a large group of wild accessions from the genus *Lens,* on a species by species basis in indoor conditions, either in growth chambers or the greenhouse. Chapter 3 was accepted for publication in Plant Genetic Resources: Characterization and Utilization on September 13, 2012.

CHAPTER 3

SCREENING OF WILD AND CULTIVATED LENTIL GERMPLASM FOR RESISTANCE TO STEMPHYLIUM BLIGHT

3.1 Introduction

Pulse crops are used with cereals in human diets around the world. Cultivated lentil (*Lens culinaris* Medik. ssp. *culinaris*) is believed to have been domesticated and consumed since pre-historic times (Sandhu and Shing, 2007). World lentil production in 2010 was estimated at 4.58 Mt on about 4.18 M ha with an average yield of 1094 kg ha^{-1} (FAOSTAT, 2010).

Lentil production is often biotically constrained by fungal diseases which cause yield instability and reduced seed quality. Stemphylium blight (SB) caused by *Stemphylium botryosum* Wallr. is a devastating disease of lentil in Bangladesh, Nepal and north-eastern India (Bakr and Ahmed, 1992; Chen et al., 2009). Reports of SB exist from Canada, Syria, Egypt, Hungary and the USA (Bayaa and Erskine, 1998; Morrall et al., 2006; Chen et al., 2009). It can cause yield losses above 80% in south Asia (Bakr and Ahmed, 1992). Results from commercial seed tests revealed high levels of SB in samples of lentil from central and northern districts of Saskatchewan, Canada (Morrall et al., 2006). No fungicides are registered specifically for the control of SB on lentil in Canada, and little is known about the efficacy of fungicides on this pathogen.

Use of disease resistant cultivars is considered the most economical approach to SB management. Two studies have been conducted on genetic control of SB resistance in lentil, both indicating that resistance to SB is quantitatively inherited (Kumar, 2007; Saha, 2009). Sarker et al., (1999) reported on the development of cultivar BARIMasur-4 with resistance to SB, first released in Bangladesh in 1996 through a collaboration of the Bangladesh Agricultural Research Institute (BARI) and the International Centre for Agricultural Research in the Dry Areas (ICARDA) in Syria (Sarker et al., 2004) followed by three more SB-resistant cultivars. Few sources of resistance to SB exist among cultivars in other lentil production regions (A. Vandenberg, unpublished data). Tullu et al. (2011) reported potential sources of

resistance to SB from the secondary and tertiary gene pool of lentil. Preliminary evidence showed high levels of resistance to SB in lines derived from hybrids between cultivated lentil and *Lens ervoides.* These were originally developed for potential introgression of resistance to ascochyta blight (caused by *Ascochyta lentis* Vassiljevsky) and anthracnose (caused by *Colletotrichum truncatum* Andrus & Moore) (Tullu et al., 2006, 2010, 2011; Fiala et al., 2009).

Interspecific hybridization in *Lens* for introgression of genes from the wild gene pool is recognized as a tool for widening the genetic base. Evidence exists for resistance in the wild gene pool for diseases and abiotic stresses (Bayaa et al., 1994; Gupta and Sharma, 2006; Tullu et al., 2010). No reports exist on systematic screening of wild lentil germplasm for SB resistance. We hypothesized that resistance to SB existed in wild lentil species and that it would be possible to identify superior sources of SB resistance for transfer to the cultivated species.

3.2 Materials and methods

3.2.1 Selection of genotypes from *Lens culinaris*

Fourteen *Lens culinaris* genotypes representing a range of adaptation were selected as a baseline for characterizing the SB reaction of cultivated lentil and were evaluated in a growth chamber (Table 3.1). Ten of the 14 genotypes were subsequently characterized in the greenhouse and field at the University of Saskatchewan in 2011. Nine of the 14 also were characterized for SB reaction in the field in the winter season of 2011-12 at the Pulses Research Centre in Bangladesh. The group included four Canadian lentil cultivars, international germplasm accessions and parents of recombinant inbred lines (RILs) developed at Crop Development Centre (CDC) of the University of Saskatchewan. For some accessions, preliminary results on their SB reaction from earlier studies under controlled condition or in the field were available (Table 3.1), whereas others were selected either on the basis of their performance against SB in foreign environments, or as a parent of newly developed recombinant inbred lines (RILs). Canadian cultivars 'Eston', a small seeded green lentil (Slinkard and Bhatty. 1981), and 'CDC Glamis', a large seeded green lentil (Vandenberg et al., 2002a), classified as resistant and susceptible checks, respectively, on the basis of their previous performance, were included in all experiments as controls.

Table 3.1. Selected *Lens culinaris* genotypes based on previous studies with published reactions to stemphylium blight, ascochyta blight, and anthracnose

Genotype[§]	Source / origin	Stemphylium blight	Ascochyta blight	Anthracnose
'Eston'	Canadian cultivar	R [1]	S [5]	S [6]
CDC Robin	Canadian cultivar	S [2]	PR [4]	PR - race 0 [6]
CDC Milestone	Canadian cultivar	MR [1]	R [7]	S [7]
CDC Glamis	Canadian cultivar	S [1]	R [8]	S [8]
VIR 421	Vavilov Institute	----	----	PR - race 0 [9]
PI 320937	IPK, Germany	----	R [5]	MR[5]
ILL 8006	ICARDA	R [1]	----	----
ILL 4605-2	Argentina	R [2]	----	----
ILL 5888	ICARDA	S [3]	----	----
ILL 8009	ICARDA	S [2]	----	----
ILL 8008	ICARDA	R [2]	----	----
ILL 5588	ICARDA	R [10]	S [5]	
ILL 7537	ICARDA	----	R [5]	----
ILL 1704	ICARDA	----	S [5]	----

[§]ILL 4605-2 is the cultivar Precoz (Argentina); ILL 5588 is the cultivar Northfield (Australia)

R= resistant, MR = moderately resistant, PR = partially resistant, S = susceptible

[1] (S. Banniza, unpublished data), [2] (Kumar, 2007), [3] (Saha, 2009), [4] (Vandenberg et al., 2002b) [5] (Tullu et al., 2010), [6] (Armstrong-Cho et al., 2012), [7] (Vandenberg et al., 2001), [8] (Vandenberg et al., 2002a) [9](Vail, 2012), [10] (A. Vandenberg, unpublished data).

3.2.2 Selection of genotypes from wild *Lens* species

A total of 56 wild species accessions were selected for the screening of SB reactions under growth chamber and greenhouse conditions, with priority given to accessions previously screened for resistance to anthracnose and ascochyta blight. Fifty-one accessions, designated with the 'IG' prefix were obtained from ICARDA and originated from diverse geographic areas of the globe with a high genetic variability at the intra and interspecies level (Tullu et al., 2006; Tullu et al., 2010). Four accessions (designated with the 'PI' prefix) were selected from among 73 wild accessions provided by the Western Regional Plant Introduction Station, USDA-ARS, Pullman, Washington, USA (Tullu et al., 2006). One accession, L-01-827A, was developed by single plant selection from *L. orientalis* accession IG 72847 and was categorized as *L. ervoides* based on morphological similarity with this species (Fiala et al., 2009). The accessions selected from *L. ervoides*, *L. culinaris* ssp. *orientalis* and

L. tomentosus were evaluated for SB resistance in the growth chambers. Accessions of *L. nigricans*, *L. odemensis* and *L. lamottei* were evaluated in the greenhouse.

3.2.3 Experiments under controlled conditions

Experiments under controlled conditions were conducted in growth chambers (Conviron Winnipeg, Manitoba) located in the College of Agriculture and Bioresources, and in greenhouses of the University of Saskatchewan. Each experiment was conducted twice. Day length was set for 16 h at 20 °C and 8 h darkness at 15 °C in growth chambers, where a cluster of 34 fluorescent tubes (PHILIPS-Silhouette high output, F54T5/835/HO/A/EA, 49W, 3500K, Alto collection Holland) provided light with approximately 400-500 μE m^{-2} s^{-1} light intensity during day time. In the greenhouse, photoperiod was maintained at 18h day and 6h night at 20-22 °C /16-18 °C (day/night) temperature. High pressure sodium lights were used to provide approximately 300-1100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) in the light period. Six seeds of each entry were planted in a 10 cm plastic pot with soil-less media (Sunshine 4 mixture, Sun Grow Horticulture, Vancouver, British Columbia, Canada). Each pot was considered a replication and four pots were used for each experiment in a randomized complete block design. For screening wild species, *L. culinaris* checks were planted one week later to synchronize the correct growth stage, pre-flowering to flowering, for inoculation. Seeds were scarified with a sharp blade before planting to ensure quick imbibition. Pots were watered after planting and then as required. After germination, plants were thinned to four per pot. A soluble mixture of N, P, and K (20:20:20) at 2 g L^{-1} water was applied once per week after emergence. Insects were controlled in the growth chamber as required using various control and repellent measures. Prior to inoculation, each pot was wrapped with transparent plastic to increase humidity around the plants.

3.2.4 Inoculation of experiments under controlled conditions

An aggressive isolate of *S. botryosum* (SB-19) was selected on the basis of previous screening of several isolates (S. Banniza, unpublished data). Sporulation of *S. botryosum* is very poor (Chowdhury et al., 1996) and laboratory preparation of spore

suspensions is difficult. Therefore, mycelial suspensions were used using a protocol previously developed at the CDC (S. Banniza, unpublished data). To prepare mycelial suspensions, stemphylium cultures were grown in 90 mm sterile Petri dishes containing V8-PDA medium (150 ml V8 Original Blend Vegetable Cocktail [Campbell Co., Canada], 10 g Difco™ Potato Dextrose Agar, 10 g Difco™ Agar, Granulated [both Becton Dickinson and Co., Sparks, MD, USA], 3 g $CaCO_3$ [EMD Chemicals Inc., Darmstadt, Germany] and 150 ml distilled water). Cultures were incubated for two weeks at 27 °C. Erlenmeyer flasks containing 500 mL modified Richard's medium (10g sucrose [Fisher Scientific, USA], 10g KNO_3 [EMD, USA], 5g KH_2PO_4 [VWR, USA], 2.5g $MgSO_4{}^*7H_2O$ [EM Science, Germany], 0.02g $FeCl_3$ [EMD, USA], 150ml V-8 juice, 850 mL distilled water, pH = 6.0) were inoculated with 10 mycelial plugs from the growing edge of colonies. Bottles were covered with aluminum foil and incubated in a rotary shaker at 110 rpm for 10 days at 20 °C. The liquid culture was vacuum-filtered and dried in sterile conditions. The dry mycelium was grounded with a coffee grinder for 1 min and kept at 4 °C in Petri dishes. The ground mycelium was blended with distilled water (1 g mycelium L^{-1} water) and 0.01% agar to maintain mycelial suspension. The suspension was diluted to obtain a spectrophotometer reading of approximately 1 when read at a wavelength of 600 nm which was equivalent to approximately 9200 colony forming units mL^{-1} suspension based on colonies counted from dilutions plated out on tap water agar (data not presented). Two drops of Tween® 20 were added to mycelial suspensions to facilitate fungal - plant tissue contact by reducing the surface tension of water, and each plant was sprayed with 2 mL of this suspension.

Plants were inoculated using an air-brush (Badger Airbrush model TC 20) at 138 kPa to evenly apply 2 mL / plant of mycelial suspension, followed by incubation in an incubation chamber at 100% humidity and 20 °C for 48 h. Plants for growth chamber experiments remained in the growth chamber where two humidifiers (VICKS ultrasonic humidifiers, Fabrique Paz Canada, Inc., 510, Milton, Ontario) were engaged for 10 min every 2 h to ensure leaf wetness until final scoring. For greenhouse experiments, plants were moved from the incubation chamber to misting benches in the greenhouse where they were misted for 60 s every hour from

6 am to 11 pm. Plants were evaluated for SB severity 15 and 21 days after inoculation.

3.2.5 Field experiments at the University of Saskatchewan, Saskatoon, 2011

A field evaluation was conducted in the summer of 2011 in fields with a dark brown, clay loam soil texture with a good water holding capacity at the University of Saskatchewan in Saskatoon (52°36′N, 106°6′W). The average maximum and minimum air temperature of Saskatoon in 2011 (May-August) was 18.4°C and 10.9°C respectively (Environment Canada, 2012). As in experiments under controlled conditions, 'CDC Glamis' and 'Eston' were used as susceptible and resistant checks, respectively. Seeds of each accession were sown with a tray hill planter as a randomized complete block design with four replications. Each hill had 4 rows of 30 cm length with 30 cm spacing between rows. A total of 20 seeds were planted in each hill. The outer rows of each hill were planted with the resistant and susceptible checks, respectively. The two centre hill rows were one of the 10 genotypes.

To inoculate and induce infection with SB, spreader plants were developed in a poly-house complex in July 2011. A random mixture of four susceptible cultivars ('CDC Glamis', ILL 5588, VIR421 and ILL 4605) was planted at 6 seeds per one-gallon pot and inoculated with a mycelial suspension as described before. Pots were covered by a low plastic tunnel and plants were incubated under high humidity for 48 h using a humidifier (Trion 356686-101 N/A Herrmidifier Atomizing Humidifier 707U). After 48 h, higher levels of humidity were maintained by running the humidifier for 20 min every 3 h for another three weeks until spreader plants were transplanted into the field. One pot of 6 spreader plants was transplanted after every third hill in each block. Following irrigation after transplanting, each block was covered with a perforated green polyethylene low tunnel to maintain higher levels of humidity. Temperature and humidity were monitored with a Hobo data logger (Onset Computer Corp., MA, USA) and plots were irrigated twice. Plants were evaluated for SB severity at 65 and 80 days after planting or 21 and 35 days after transplanting spreader plants.

3.2.6 Field experiments at PRC, Ishurdi, Bangladesh, 2011-12

In Bangladesh, field experiments were conducted at the PRC of BARI at Ishurdi. A completely randomized block design experiment was established with four replicates. A highly susceptible local cultivar BARIMasur-1 (ILL 5888) and the resistant cultivar BARIMasur-4 (ILL 8006) were used as susceptible and resistant checks, respectively. Two Canadian cultivars 'Eston' and 'CDC Glamis' were also used as resistant and susceptible checks, respectively. Each line was planted in a one-meter row plot with a row of ILL 5888 planted adjacent at 30 cm distance. Each block was surrounded by ILL 5888 that served as spreader plants. Plant debris collected from highly infected plants from the 2010-11 field season was crushed and spread in plots at the vegetative to pre-flowering stage as a source of inoculum. Disease severity was assessed 120 days after seeding.

3.2.7 Disease scoring and statistical analysis

Plants in all experiments were evaluated using a 0-10 scale where 0 = 0% disease severity (DS), 1= 1-10% DS, and so forth, up to 10 = 91-100% DS. All statistical analyses were conducted with SAS version 9.2 (SAS institute Inc., Cary, NC, USA). Data were transformed into percentage data using the midpoint of each disease class, and tested for homogeneity of variance with the Levene's test for homogeneity. Data analysis was done using the mixed model procedure where accessions were considered a fixed factor and replicates a random factor. Means were separated using Fisher's least significant difference. After analysis, genotypes were categorized into five groups. Genotypes more resistant to 'Eston' were considered very resistant (VR), those equal to 'Eston' resistant (R). Genotypes with DS similar to CDC Glamis were susceptible (S), those with significantly more stemphylium blight very susceptible (VS). Genotypes with higher DS than 'Eston' and lower DS than 'CDC Glamis' were considered intermediate (I).

3.3 Results

3.3.1 Disease severity ratings of *Lens culinaris* genotypes

Disease severity in the experiments under controlled and field conditions ranged from 25% (ILL 8006) to 54% (PI 320937) in the growth chamber, 24% (ILL 1704) to 69% (ILL 5888) in the greenhouse, 15% (ILL 8006) to 67% (ILL 5888) in the field at the

Pulses Research Centre, Ishurdi, Bangladesh, and from 25% ('Eston') to 62.50% (CDC Glamis) in the field at Saskatoon (Table 3.2).

Table 3.2. Stemphylium blight percent disease severity (DS) and disease reaction of *Lens culinaris* genotypes in four different environments.

Genotype	Growth chamber		Greenhouse		Field, Saskatoon Canada		Field, Ishurdi Bangladesh	
	Mean DS %	Std. error	Mean DS %	Std. error	Mean DS %	Std. error	Mean DS%	Std. error
CDC Glamis	54 (S)	3.1	62 (S)	1.9	63 (S)	2.5	48 (S)	3.3
CDC Milestone	38 (I)	1.8	33 (R)	1.5	38 (I)	2.5	35 (I)	7.0
CDC Robin	40 (I)	2.5	41 (I)	3.0	45 (I)	4.0	38 (S)	2.5
Eston	24 (R)	1.4	29 (R)	1.3	25 (R)	4.0	23 (R)	6.2
ILL 1704	24 (R)	1.0	24 (R)	1.9	35 (I)	4.0	30 (R)	2.8
ILL 4605-2	48 (I)	2.0	39 (I)	2.2	55 (S)	4.0	20 (R)	2.8
ILL 5588	53 (S)	2.1	44 (I)	3.0	58 (S)	3.3	--	--
ILL 5888	54 (S)	3.1	69 (VS)	1.1	60 (S)	2.8	68 (VS)	2.5
ILL 7537	43 (I)	1.4	45 (I)	2.0	--	--	--	--
ILL 8006	25 (R)	1.7	30 (R)	2.5	53 (S)	2.5	15 (R)	0.0
ILL 8008	46 (I)	1.5	39 (I)	2.7	--	--	--	--
ILL 8009	57 (S)	2.5	50 (I)	2.5	--	--	--	--
VIR 421	53 (S)	2.0	53 (I)	1.6	--	--	--	--
PI 320937	60(VS)	2.0	50 (I)	1.6	60 (S)	2.8	48 (S)	2.5

Disease reactions: (R)- similar to 'Eston'; (S)-similar to CDC Glamis; (VS)-more susceptible than CDC Glamis; (I) intermediate between 'Eston' and CDC Glamis.

Significant differences in SB severity were observed among the genotypes under controlled and field conditions (Appendix 1-4 and 10). A significant difference for disease severity was observed between both the resistant and susceptible checks in all experiments. None of the genotypes showed VR disease reaction from all environments. Most, but not all *L. culinaris* parents showed consistency in disease reactions under the different test conditions. For example, 'Eston' showed a consistent resistant reaction in all environments with a DS score ranging from 23 to 29%. Variable results were also observed, e.g. the genotype ILL 1704 had similar levels of resistance compared to 'Eston' in all tests with the exception of the field experiment at Saskatoon where disease severity was significantly higher for ILL 1704 than for

'Eston' (Table 3.2). The highly susceptible line ILL 5888 from Bangladesh showed VS reaction in the greenhouse and under field conditions in Bangladesh, and S in the other two environments. The highest number of intermediate reactions was observed under greenhouse conditions whereas most of those genotypes would display a susceptible reaction under the other testing conditions. Maximum susceptible reactions in genotypes were observed in the field at Saskatoon followed by tests in the growth chamber.

3.3.2 Disease severity ratings of wild *Lens* species

Typical symptom development 3-4 days after inoculation on susceptible *L. culinaris* checks confirmed the virulence of isolate SB-19. Significant differences for DS were observed within accessions from each wild species (Appendix 5-9 & 11). Mean DS values for 'Eston' (28%) and 'CDC Glamis' (62 %) were consistent and significantly different from each other in all experiments. Among 56 accessions from the six wild species, a high number of accessions were found with resistance to SB as good as or better than the resistant check 'Eston' (Tables 3.3, 3.4 and 3.5). No wild species accessions had higher DS than the susceptible check 'CDC Glamis'.

Table 3.3. Disease severity (DS) percentage for accessions of *Lens ervoides* and *Lens nigricans* inoculated with *Stemphylium botryosum* isolate SB-19, and disease reactions (DR) to ascochyta blight and anthracnose in previous studies.

Accessions	Origin	ILWL No.	Stemphylium blight			Ascochyta blight DR	Anthrac-nose DR
			Mean DS %	Std. error	DR		
Lens ervoides							
IG 72646	Syria	123	23	3.6	R	R [3]	R [1]
IG 72651	Syria	128	28	2.8	R	R [2]	R [1]
IG 72654	Syria	131	30	4.0	R	R [3]	R [1]
IG 72799	Turkey	276	24	1.4	R	R [3]	R [1]
IG 72803	Turkey	280	22	3.4	VR	--	R [1]
IG 72815	Turkey	292	25	1.4	R	R [3, 4]	R [1]
IG 116033	Turkey	461	16	3.7	VR	--	R [1]
IG 107435	Syria	406	44	5.7	I	S [3]	R [1]
IG 107441	Syria	413	36	1.9	R	S [3]	R [1]
L 01-827A [5]	Canada	--	22	2.8	VR	R [5]	R [1]
'Eston' [§]	Canada	--	25	4.3	R	S [3]	S [7]
CDC Glamis [§]	Canada	--	56	3.4	S	R [6]	S [6]
Lens nigricans							
IG 72539	France	16	40	1.7	R	MR [3]	--
IG 72547	Unknown	24	25	4.1	R	R [3]	--
IG 72548	Turkey	25	23	4.4	VR	--	--
IG 72549	Unknown	26	35	3.4	R	--	--
IG 72550	Ukraine	27	33	5.2	R	--	MR [1]
IG 72551	Unknown	28	14	1.5	VR		MR [1]
IG 72553	Spain	30	22	3.0	VR	R [3]	--
IG 72557	Ukraine	34	25	3.0	VR	R [3]	R [1]
IG 72560	Turkey	37	32	2.4	R	R [3]	--
IG 72633	Turkey	110	38	1.5	R	R [3]	
IG 72713	Turkey	190	21	1.0	VR	R [2,3]	MR [1]
IG 72795	Turkey	272	27	2.0	R	R [3]	--
IG 72843	Turkey	320	18	0.7	VR	--	--
IG 116018	Turkey	446	29	3.3	R	MR [3]	--
IG 116024	Turkey	452	49	1.9	I	R [3]	
IG 136636	Unknown	--	42	2.1	R	--	--
IG 136641	Unknown	--	49	2.8	I	--	--
IG 136645	Unknown	--	24	4.2	VR	--	--
'Eston' [§]	Canada	--	34	2.1	R	S [3]	S [7]
CDC Glamis [§]	Canada	--	71	1.8	S	R [6]	S [6]

For stemphylium blight: VR-more resistant than 'Eston'; R- similar to 'Eston'; S-similar to CDC Glamis; VS-more susceptible than CDC Glamis; (I) intermediate between 'Eston' and CDC Glamis. [§] *L. culinaris* controls; MR = moderately resistant; DR = Disease reaction; [1](Tullu, et al. 2006), [2] (Bayaa et al. 1994), [3] (Tullu, et al. 2010), [4] (E. Sari, University of Saskatchewan, Canada, personal communication), [5](Fiala et al., 2009), [6] (Vandenberg et al., 2002a), [7] (Armstrong-Cho et al., 2012)

Table 3.4. Disease severity (DS) percentage for accessions of *Lens c.* ssp. *orientalis* and *Lens tomentosus* inoculated with *Stemphylium botryosum* isolate SB-19, and disease reactions (DR) to ascochyta blight and anthracnose in previous studies.

Accessions	Origin	ILWL No.	Stemphylium blight			Ascochyta blight DR	Anthra-cnose DR
			Mean DS (%)	Std. error	DR		
L. culinaris* ssp. *orientalis							
IG 72622	Turkey	99	13	1.7	R	R [3]	--
IG 72642	Syria	119	24	5.2	R	R [3]	--
IG 72829	Turkey	306	10	1.4	VR	R [3]	--
IG 72611	Turkey	88	13	1.3	R	R [2, 3]	--
IG 72592	Turkey	69	12	1.7	R	R [2, 3]	--
IG 72907	Tajikistan	384	15	1.9	R	S [3]	--
IG 72905	Tajikistan	382	26	4.6	R	R [3]	--
IG 110824	Lebanon	421	20	3.0	R	--	--
PI 572385	Turkey	--	13	1.4	R	S	--
PI 572390	Turkey	--	48	5.0	S	S	--
PI 572379	Turkey	--	36	4.0	I	S	--
PI 572375	Israel	--	43	3.3	S	S	--
§'Eston'	Canada	--	21	1.7	R	S [3]	S [1]
§CDC Glamis	Canada	--	53	2.3	S	R [4]	S [4]
Lens tomentosus							
IG 72613	Turkey	90	36	9.7	I	--	--
IG 72643	Turkey	120	11	1.4	VR	--	--
§'Eston'	Canada	--	22	1.8	R	S [3]	S [1]
§CDC Glamis	Canada	--	55	2.3	S	R [4]	S [4]

For stemplylium blight: VR-more resistant than 'Eston'; R- similar to 'Eston'; S-similar to CDC Glamis; I intermediate between 'Eston' and CDC Glamis. DR = Disease reaction;

§ *L. culinaris* controls. [1](Armstrong-Cho et al., 2012), [2] (Bayaa et al. 1994), [3] (Tullu, et al. 2010), [4] (Vandenberg et al., 2002b)

Table 3.5. Disease severity (DS) percentage for accessions of *Lens lamottei* and *Lens odemensis* inoculated *Stemphylium botryosum* isolate SB-19, and disease reactions (DR) to ascochyta blight and anthracnose in previous studies.

Accessions	Origin	ILWL No.	Stemphylium blight			Ascochyta blight disease reaction	Anthra-cnose disease reaction
			Mean DS (%)	Std. error	DR		
Lens odemensis							
IG 72543	Palestine	20	28	2.4	R	R [2]	--
IG 72606	Turkey	83	20	1.6	VR	R [2]	--
IG 72623	Turkey	100	30	2.2	R	R [2]	--
IG 72639	Syria	116	37	2.6	I	--	--
IG 72676	Syria	153	43	2.5	I	MR [2]	--
IG 72693	Syria	170	52	2.5	I	R [2]	--
IG 72745	Turkey	222	24	2.1	R	MR [2]	--
IG 72777	Syria	254	25	4.5	R	R [2]	--
IG 116008	Turkey	436	33	1.7	R	MR [2]	--
Lens culinaris controls							
Eston	Canada	--	32	1.1	R	S [2]	S [4]
CDC Glamis	Canada	--	63	1.2	S	R [3]	S [3]
Lens lamottei							
IG 72537	France	14	14	1.0	VR	--	--
IG 72552	Spain	29	14	2.4	VR	--	MR-race 1 [1] S - race 0 [1]
IG 110809	Spain	428	16	2.6	VR	--	R [1]
IG 110810	Spain	429	22	3.1	R	--	MR [1]
IG 110813	Spain	432	21	3.1	R	R [2]	R - race 1 [1] S - race 0 [1]
Lens culinaris controls							
Eston	Canada	--	26	1.1	R	S [2]	S [4]
CDC Glamis	Canada	--	62	2.1	S	R [3]	S [3]

VR-more resistant than 'Eston'; R-similar to 'Eston'; S-similar to CDC Glamis; VS-more susceptible than CDC Glamis; I- intermediate between 'Eston' and CDC Glamis. MR = moderately resistant; DR = Disease reaction; [1] (Tullu, et al. 2006), [2] (Tullu, et al. 2010), [3] (Vandenberg et al., 2002b), [4] (Armstrong-Cho et al., 2012)

Among *L. ervoides*, accessions L-01-827, IG 116033 and IG 72803 had significantly lower DS than the resistant check 'Eston' and were classified as VR, whereas

accessions IG 72651, IG 72815, IG 72646, IG 72799, IG 72654 and IG 107441 had resistant (R) reactions similar to 'Eston' (Table 3.3). The remaining accession IG 107435 had DS significantly higher than 'Eston' and lower than 'CDC Glamis' thus was considered intermediate. Among the 18 accessions from *L. nigricans,* seven fell into the VR category, and nine in the R category (Table 3.3). The remaining two accessions displayed intermediate reactions, and none of the accessions showed higher DS than 'CDC Glamis'.

One accession (IG 72829) out of 12 from *L. c.* ssp. *orientalis* showed VR disease reactions, and two accessions (PI 572375 and PI 572390) were susceptible to SB with similar DS score as CDC Glamis (Table 3.4). Among the remaining nine accessions, eight were resistant and one was in intermediate in its reaction to SB. The two *L. tomentosus* accessions IG 72643 and IG 72613 had an R and I reaction, respectively (Table 3.4). Three accessions out of nine from *L. odemensis* (IG72639, IG 72676 and IG 42693) had intermediate scores to SB; five accessions showed resistance to SB and only one accession (IG 72606) had significantly lower DS than 'Eston' (Table 3.5). Among the five *L. lamottei* lines, IG 72552, IG 72537 and IG 110809 were VR and the remaining two were resistant to SB (Table 3.5).

The highest percentage of resistant accessions was found in *L. lamottei* (100%) followed by *L. ervoides* (90%) and *L. nigricans* (88%). The other three species, *L. c.* ssp. *orientalis L. odemensis,* and *L. tomentosus* had 75%, 70% and 50% resistant accessions, respectively (Figure 3.1).

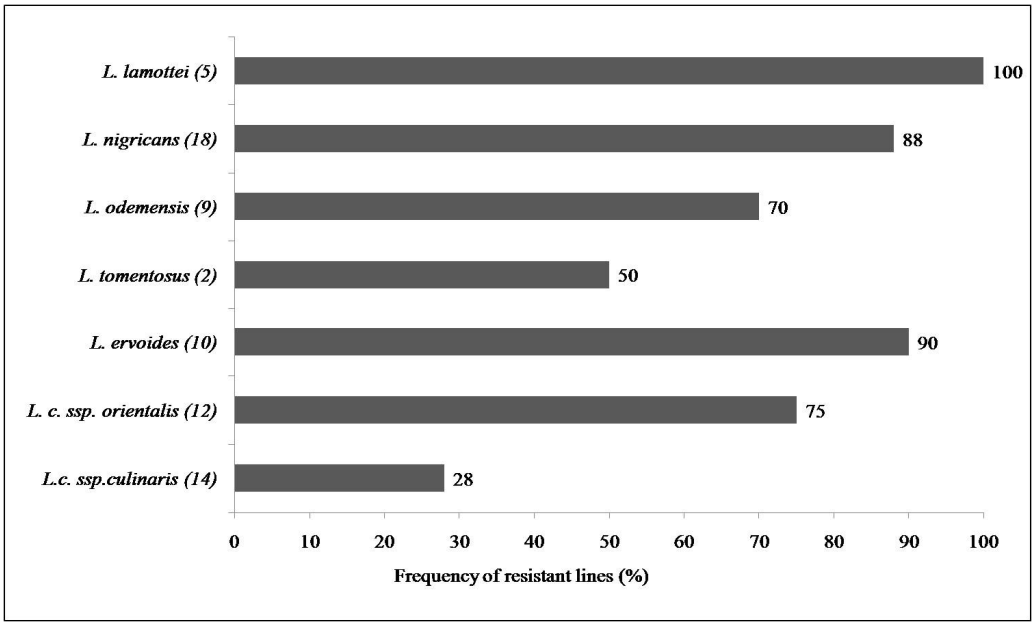

Figure 3.1. Percentage of resistant accessions of seven *Lens* species inoculated with *Stemphylium botryosum* isolate SB-19 under phytotron, greenhouse and field conditions of Saskatoon and in the field of Bangladesh under natural conditions. Number of accessions evaluated in brackets after each species name.

3.4 Discussion

Detailed screening of *L. culinaris* genotypes and accessions of wild lentil species confirmed that frequency of resistance to SB in domesticated lentil is limited, but it occurs frequently in all wild species. The high frequency of resistance to SB in the wild lentil species is likely due to the much higher level of genetic variability in these populations compared to the cultivated species in which the gene pool probably has been narrowed through centuries of selection and breeding. A long history of co-evolution between *S. botryosum* and wild lentil species in the centre of origin most likely contributed to the high abundance of resistance in these wild populations, too. Indeed, *S. botryosum* has been reported from Syria (Hanounik, 1979), which is believed to be part of the centre of origin for cultivated lentil (Cubero et al., 2009). Ten of the 56 wild species accessions originated from Syria and another 22 from the geographic neighbour Turkey, and all of the *L. ervoides* accessions from these two countries had a higher frequency of resistance. Bayaa et al., (1994) and Tullu et al.,

(2006, 2010) reported similar results for ascochyta blight and anthracnose resistance. Almost all the accessions from *L. orientalis*, *L. nigricans* and *L. odemensis*, which are considered as "most likely candidates of the cultigens" (Cubero et al., 2009) showed highly resistant reactions to SB, and are of similar geographic origin as *L. ervoides*. These results support the hypothesis that species in the centre of origin are characterized by high levels of diversity, including disease resistance. Leppik (1970) reviewed sources of resistance with regard to their origin for a range of crop species and demonstrated that the primary or secondary centre of origin represented a valuable source of genotypic diversity for resistance to different biotic stresses.

Different morphological features among the different species compared to domesticated lentil may influence the ability of pathogens like *S. botryosum* to invade the lentil. Highly significant differences between *L. culinaris* and other wild species were reported by Hoffman et al. (1998) for number of leaflets, leaflet length and width, and leaf pubescence. Significant morphological and phenological variation between cultivated and wild *Lens* species was also observed by Ferguson and Robertson (1999), which was also related with different growth stages and disease development. For example, SB usually infects plants from flowering onwards (Kumar, 2007). Chowdhury et al. (1997) also reported on the role of epidermal hairs, thickness of epidermis, cortical layer depth and number of stomata in disease resistance of cultivated lentil.

Some wild accessions had little infection, restricted to tiny lesions after a few days of inoculation, which did not expand further. Both susceptible and resistant accessions were found from all species except *L. lamottei*. In this study, all accessions of *L. lamottei* were resistant to SB. The highest number of accessions with resistance to *C. truncatum*, both under greenhouse and field conditions, was found in *L. ervoides* (Tullu et al., 2006). The authors also identified good resistance to the more aggressive race 0 of *C. truncatum* among accessions of *L. ervoides* and *L. lamottei*, resistance that is not present in *L. culinaris*. Twenty-five wild lentil accessions among those reported as resistant to ascochyta blight by Tullu et al., (2010) were also identified as resistant to SB in this study (Tables 3.3, 3.4 and 3.5). These results could be of significant importance for lentil improvement programs because all three diseases are

now a major concern for lentil cultivation in the northern temperate prairies of North America. Based on agro-climatic similarity, it can be expected that stemphylium blight will also become a concern in northern temperate regions of Asia where lentil production is expanding. Availability of superior resistance to all three pathogens in the same germplasm will simplify the development of cultivars with triple resistance through single transfer of the resistance genes into cultivated lentil.

Searching for SB resistance sources within cultivated lentil was the initial goal of research related to breeding for resistance. However, the highest percentage of susceptibility to SB was found in *L. c.* ssp. *culinaris* under all conditions. Kumar (2007) reported very poor resistance to SB and inconsistent disease reactions in different environments among accessions of *L. culinaris*. In our experiments, consistent SB resistance was displayed by cultivar 'Eston', but this cultivar is highly susceptible to both ascochyta blight and anthracnose. Furthermore, it is desirable to use diverse sources of resistance in lentil breeding programs to achieve durability of disease resistance. Several factors, such as higher selection pressure, poor management practices, narrow genetic base, rapid evolution of the pathogen population, changing climate and other environmental factors can influence the breakdown of resistance in the cultivated species. Consequently, the high frequency of resistant accessions among wild accessions with higher resistance than in *L. culinaris* is very promising for future genetic improvement efforts.

Resistance sources from cultivated and wild species identified in this study can be used in intra and interspecific crosses to develop adapted cultivars with SB resistance. By strategically selecting accessions with multiple disease resistance for this purpose, resistance to SB can be incorporated into the lentil breeding program to develop new cultivars with disease resistance to SB, ascochyta blight and anthracnose, as suggested by Tar'an et al., (2003). Research is underway at the Crop Development Centre to develop lentil cultivars with genetic resistance to SB as part of a long-term strategy for reducing potential economic losses caused by the disease. Special techniques are required to obtain fertile hybrids from interspecies crosses (Fiala et al., 2009). Techniques include tissue culture for multiplication of F_1 plants from rescued embryos (Shyamali Saha, Crop Development Centre, University of Saskatchewan,

Canada, unpublished data), grafting to faba bean (Yuan et al., 2011) and reproduction using cuttings for multiplication to achieve rapid disease screening (Vail, 2010) have been successfully established and have made it feasible to reliably transfer resistance from wild to cultivated lentil.

3.5 Conclusions

Results from the current study contribute scientific knowledge that will help with the development of an effective breeding strategy for SB resistance in lentil. Some wild species accessions having significantly lower DS than 'Eston' can be given higher priority for future breeding efforts. Genetic characterization of sources of SB resistance across the genus *Lens* combined with the development and use of genomics-based marker assisted selection techniques for breeding are important milestone for utilization. Moreover, considering that all wild *Lens* species have good sources of resistance to SB, that the most accessible sources are in the primary gene pool consisting of *L. c.* ssp. *orientalis, L. tomentosus* and *L. odemensis*, and that these species are rich sources of multiple resistance to fungal diseases, credence should be lent to current efforts to collect and preserve these valuable sources of genetic diversity.

3.6 Prologue to Chapter 4

Identification of resistance sources for SB from different *Lens* species, mainly from wild species, encouraged to study the inheritance of resistance to SB. The results from multi-location screening of *L. culinaris* accessions found that 'Eston' and PI 320937 were consistently resistant and susceptible to SB, respectively. The intraspecific RIL population LR-39, derived from a cross between 'Eston' and PI 320937 was chosen for screening for SB resistance. The LR-39 population had 96 six F_7 derived F_8 RILs and was large enough to phenotype for SB resistance. Chapter 4 presents the results of the analysis of SB resistance across environments for LR-39. This work provides a framework for understanding the genetics of inheritance of SB resistance in intraspecific lentil hybrids. The study is presented in manuscript format for submission to a scientific journal in the fall of 2012.

CHAPTER 4

SCREENING OF INTRASPECIFIC RECOMBINANT INBRED LINES OF LENTIL FOR RESISTANCE TO STEMPHYLIUM BLIGHT

4.1 Introduction and Objectives

Intensive study of SB has become very relevant as this disease has been affecting the newly developed lentil growing areas in the temperate northern prairies of North America. The genetics of inheritance of resistance to SB of lentil has not yet been satisfactorily explored (Saha, 2009), as is the case with ascochyta blight and anthracnose. An inheritance study for SB resistance was first reported by Kumar (2007) with RILs developed from an intraspecific cross between two *L. culinaris* parents (BARIMasur-4 × CDC Milestone) and quantitative inheritance of resistance was identified. In a study to determine the inheritance of and linkage map positions of genes conferring resistance to stemphylium blight in lentil, Saha, (2009) found a complex inheritance pattern after screening a 206 F_7- derived RIL population developed by crossing the two *L. culinaris* parent ILL 6002 (resistant) and ILL 5888 (susceptible) at PRC, Ishurdi, Bangladesh. He reported one and three significant quantitative trait loci (QTL) based on disease scores from experiments in 2006-2007 and 2008-2009, respectively, where one QTL ($QLG4_{80\text{-}81}$) was common in both the years.

An initial evaluation of *Lens culinaris* identified a few SB resistant sources from the cultivated species (Chapter 3). 'Eston' was identified as resistant to SB while PI 320937 was considered very susceptible, susceptible or intermediate depending on the environments (Table 3.2). The intraspecific RIL population LR-39 was chosen for SB screening on the basis of these results and because its previous use in studies of inheritance of resistance and identification of QTLs linked to resistance genes for two other major lentil diseases, anthracnose (Tullu et al., 2003) and ascochyta blight (Tullu et al., 2006). In these studies, 'Eston' was susceptible and PI 320937 was resistant to both these diseases. An intraspecific linkage map was constructed for LR-39 to identify the genome positions for two different quantitative trait loci for earliness and plant height of lentil (Tullu et al., 2008).

No previous studies have reported on the inheritance of SB resistance for RILs in two completely diverse field environments where SB is similarly important. Previous studies confirmed the highly conducive environment for SB screening in Bangladesh. Both parents of LR-39 are adapted to the northern temperate regions encompassing the northern grain belt of North America, so the use of adapted parent x adapted parent crosses was expected to help elucidating the inheritance pattern of resistance to SB without the confounding effect of non-adapted germplasm (Kumar, 2007). Use of diverse environments also may confirm the inheritance pattern of resistance and reveal the contribution of the environment to the phenotypes of RILs. The scientific knowledge from this study will help to develop the breeding strategy for SB resistance in lentil.

4.2 Materials and Methods

4.2.1 Field screening of the LR-39 RIL population for disease severity of SB in the field at the University of Saskatchewan in 2011

A set of 96 $F_{7:}F_8$ RILs were selected randomly from the LR-39 RIL population which had previously been developed by crossing 'Eston' and PI 320937 followed by a single-seed descent. 'Eston' is an early maturing, small green lentil cultivar (Slinkard and Bhatty, 1981). It is susceptible to *Colletotrichum truncatum* (Armstrong-Cho et al., 2012) and *Ascochyta lentis* (Tullu et al., 2010), and displayed a resistant disease reaction for SB in other experiments (Chapter 3). Parent PI 320937 is a late maturing genotype from Germany and was used as resistance source for anthracnose and ascochyta blight (Tullu et al., 2003; Tullu et al., 2006).

This experiment was conducted on the same research land used to screen *Lens culinaris* genotypes adjacent to the campus of the University of Saskatchewan in Saskatoon (Chapter 3). The methods for planting the seeds were the same as described in section 3.2.5, with the exception that the two centre hills were RILs from LR-39. An unadapted, but known susceptible check from Bangladesh (ILL 5888) was also included once in each replicate in the same arrangement as the LR-39 lines. To induce SB in the field, an artificial inoculation with spreader plants was implemented as used

in *L. culinaris* screening in the experiments described in section 3.2.5. Preparation of inoculum and procedures for inoculating spreader plants were also described in the Materials and methods section of Chapter 3.

4.2.2 Field screening of LR-39 RILs for SB disease severity at the Pulses Research Centre, Ishurdi, Bangladesh, 2011-12

As a winter (October to March) crop, lentil is seeded in Bangladesh from late October to early November. The same set of LR-39 RILs screened at Saskatoon in the summer of 2011 was evaluated for SB reaction under field conditions at the Pulses Research Centre (PRC) of the Bangladesh Agricultural Research Institute (BARI), Ishurdi. Screening procedures were the same as that described in Section 3.2.6.

4.2.3 Disease scoring and statistical analysis

Percentage disease severity (DS) was rated at 80 and 120 days after sowing in the field experiments at Saskatoon and at PRC, respectively. Disease scoring and analysis was similar to that described in section 3.2.7. Estimates were used to produce frequency distributions to study the inheritance of SB resistance. After statistical analysis, all LR-39 RILs were categorized into five groups. Lines with significantly higher resistance to SB compared to 'Eston' were considered very resistant (VR), those equal to 'Eston' were rated as resistant (R). Lines with DS similar to CDC Glamis were susceptible (S); those with significantly greater DS than CDC Glamis were considered very susceptible (VS). Lines with higher DS than 'Eston' and lower than 'CDC Glamis' were considered intermediate (I). Pooled data analysis was done to estimate the contribution of the RIL × location interaction to the total variation.

4.3 Results

All 96 F_7-derived RILs emerged at Saskatoon and at PRC in Bangladesh developed well, allowing reliable DS assessment. Significant differences ($P < 0.001$) for DS among LR-39 RILs at both locations confirmed the presence of R and S lines and segregation of resistance in the RIL population (Appendix 12-13).

4.3.1 Results of field screening of LR-39 RILs for SB disease severity at the University of Saskatchewan Saskatoon, 2011

The average maximum (18.4°C) and minimum (10.9°C) air temperature at Saskatoon for the 2011 cropping season (May–August) was favorable for lentil growth and development. Optimal environmental conditions for SB development were achieved in the low tunnel with 34°C maximum, 14 °C minimum and a mean temperature of 24°C, and an average 91% RH from 4th August to 4th September (Appendix 18).

Stemphylium blight was successfully established through the spreader plants, and mean DS (%) ranged from 16.7 % (LR-39-73) to 68.7% (LR-39-50). The parents 'Eston' and PI 320937 had DS of 33.8% and 55.0 % respectively, and were significantly different. The susceptible check 'CDC Glamis' with 66.3% DS also had significantly higher DS compared to 'Eston' but was similar to PI 320937. A group of 19 RILs showed nominally, but not significantly, lower mean DS compared to the resistant parent 'Eston' with the exception of LR-39-73 which was lower and was rated as VR. A total of 55 lines had similar DS compared to 'Eston' i.e., were considered resistant. No RILs had higher DS than CDC Glamis, but 35 had similar DS and were considered susceptible. Among the entire set of RILs, only 5 lines had statistically higher DS than 'Eston' and lower DS compared to 'CDC Glamis'. These were grouped in the I class. The disease scores of RILs were not normally distributed and slightly skewed towards resistance (Figure 4.1A).

4.3.2 Results of field screening of LR-39 RILs at PRC, BARI, Ishurdi, Bangladesh

The weather at Ishurdi was dry with a negligible rainfall in January (24 mm), none in February and a trace in March (3 mm). Stemphylium blight first appeared in early January when temperatures remained moderate to warm with cloudy days and high relative humidity. The average temperature of 21°C, relative humidity of 86% and light showers in early January were optimal for SB initiation (Appendix 18). Temperature and relative humidity remained conducive for disease progress until March.

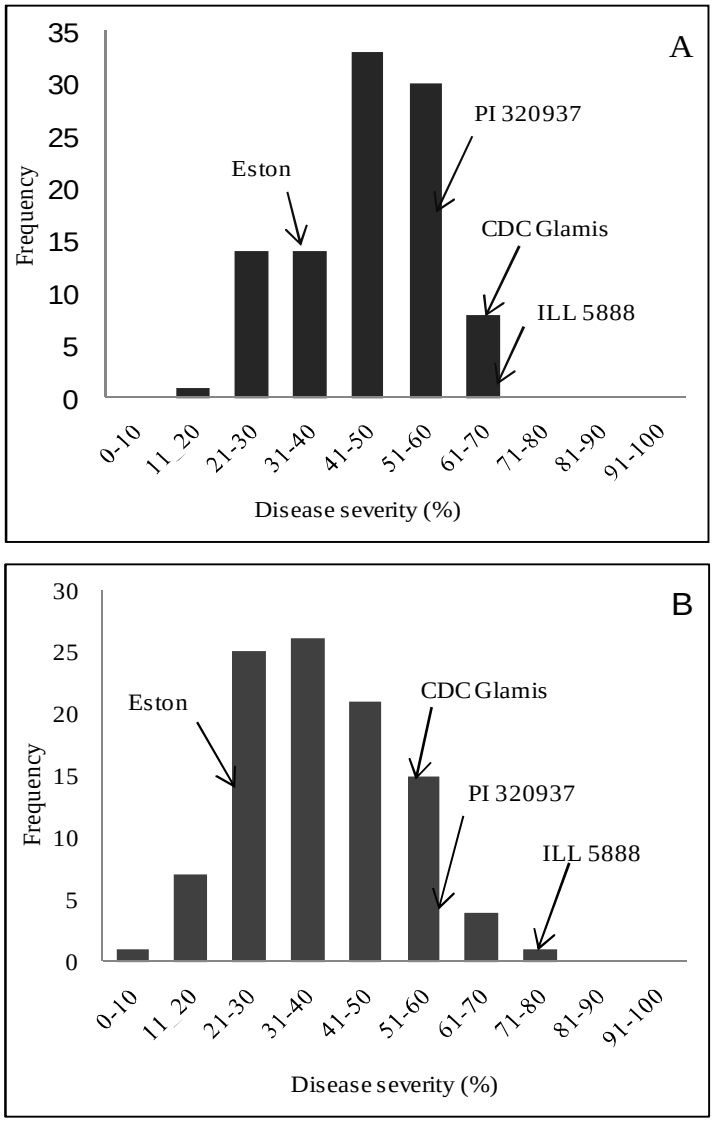

Figure 4.1. Distribution of mean SB severity scores of LR-39 RILs. (A) - at the University of Saskatchewan inoculated with spreader plants infected with *Stemphylium botryosum* isolate SB-19 in 2011, and (B) - under field conditions at the Pulses Research Centre, Ishurdi, Bangladesh in 2012 inoculated with stemphylium blight infected plant debris.

Disease symptoms in the highly susceptible check ILL 5888 and some of the RILs appeared 10 days after inoculation with plant debris. No symptoms of other diseases occurred during the entire season, providing excellent conditions for scoring DS of stemphylium blight. All local and Canadian checks and the two parents that were randomly sown four times in each replication showed consistent responses to SB

infection. A wide range of DS was observed among checks and the RILs of LR-39, from 7.5% (LR-39-89) to 67.5% (ILL 5888) and was significantly different (Appendix 16). A significant difference was found between local susceptible check ILL 5888 and both unadapted checks 'CDC Glamis' (DS = 55.0 %) and 'Eston' (DS = 27.5%), which were also significantly different from each other. None of the RILs had significantly higher DS than ILL 5888, whereas six RILs (LR-39-10, LR-39-62, LR-39-91, LR-39-21, LR-39-28 and LR-39-57) had similar DS compared to ILL 5888. One (LR-39-89) had significantly and 17 RILs had nominally lower DS than 'Eston'. A total of 31 and 28 RILs were resistant (similar DS compared to 'Eston') and susceptible (similar DS compared to 'CDC Glamis'), respectively. The remaining 35 lines had intermediate DS compared to both 'Eston' and 'CDC Glamis'. No normal distribution was observed for the disease scores of RILs as shown in Figure 4.1B.

4.3.3. Pooled analysis with both locations data

Pooled data analysis from both environments for estimating RIL × location interactions revealed heterogeneous variance for DS of variables (Appendix 16), lines and locations for the LR-39 RIL population. Transformation of data did not stabilize variances and modeling the variances using the mixed procedure was technically not possible. To obtain an approximate estimate of the contribution of line, location and their interaction to the total variability, the analysis was conducted without modeling variances. The contributions of line and location to the variation were only 7.0 % compared to 26.3% for the line × location interaction. Among the 96 lines, both consistent and inconsistent disease reactions at the two locations were observed for all five disease classes (Table 4.1). The parents of LR-39 and two the Canadian checks showed consistent disease reaction in both locations. The susceptible check from Bangladesh, ILL 5888 was VS at Bangladesh but S at Saskatoon. Lines with consistently R reactions to SB in both locations are shown in appendix 19.

Table 4.1. Consistency in disease reactions of *Lens culinaris* recombinant inbred lines (RILs) from the population LR-39 tested for stemphylium blight resistance at Saskatoon and at the Pulses Research Centre, Ishurdi, Bangladesh.

Disease reaction	No. of LR-39 RILs
R at both locations	23
I at both locations	3
S at both locations	13
R at Saskatoon but S at Bangladesh	13
S at Saskatoon but R at Bangladesh	8
I at Saskatoon but S at Bangladesh	2
R at Saskatoon but I at Bangladesh	19
S at Saskatoon but I at Bangladesh	13
VR at Saskatoon but S at Bangladesh	1
VR at Bangladesh but S at Saskatoon	1
Total	96

VR = very resistant, R= resistant, I= intermediate, S= susceptible and VS= very susceptible

4.4 Discussion

The significant phenotypic variation among the lines of the large intraspecific population LR-39, both in Bangladesh and Saskatoon indicated segregation of resistance genes among the RILs. Comparing the two locations, highest scores and diversity for DS were observed in the experiment at PRC in Bangladesh. The highest number of resistant lines was found at Saskatoon (55) compared to 31 in Bangladesh. A large number of lines (35) fell into the intermediate class based on data from Bangladesh compare to only 5 at Saskatoon. However, the mean DS at PRC was lower at 38.8 ± 19.2 compared to 45.9 ± 15.4 at Saskatoon.

About 40% of the LR-39 lines had consistent result either in resistant or intermediate or susceptible class and the remained showed inconsistent disease reaction in both locations. The frequency distributions of SB disease scores from both environments was continuous, suggesting the involvement of more than one gene, either major or minor, in controlling SB resistance as reported by Kumar (2007). Quantitatively inherited traits may be influenced by non-allelic interactions such as environmental effects that influence phenotypic expression (Saha, 2009). While studying the inheritance of anthracnose resistance in LR-39, Tullu et al. (2003) observed resistant and susceptible RILs in a 1:1 ratio, with some partially resistant and susceptible RILs.

They concluded that the resistance was due to the contribution of a major gene with a positive effect of some minor resistance genes. Evidence for the involvement of multiple genes was also reported by Netzer et al., (1985) for *S. botryosum* resistance in lettuce (*Lactuca sativa* L.).

In a study of disease reactions to SB in all the species of lentil (Chapter 3), 'Eston' and PI 320937 were found to be consistently resistant and susceptible, respectively, in four different environments in 2011 and 2012, including growth chambers, greenhouse, and field conditions at Saskatoon and Bangladesh (Chapter 3). The former and latter parents were also susceptible and resistant, respectively to both ascochyta blight and anthracnose. The results of this study have provided an opportunity to eventually explore the genetic relationship of resistance to SB and the other two major lentil diseases at the genomic level, particularly considering that a linkage map for LR-39 is available (Tullu et al., 2008).

Inconsistent stemphylium blight DS were observed in both locations for a large group of LR-39 RILs. For instance, LR-39-73 showed VR disease reaction in the field at Saskatoon but S in the field of Bangladesh, and the opposite result was observed for LR-39-89 (Appendix 19). This inconsistency between two locations could have several possible causes. Environmental factors can influence the host pathogen interaction and disease expression of quantitatively inherited diseases. The presence of locally diverse but distinct isolates with different aggressiveness in a specific location can also significantly influence disease reaction of germplasm. Significant differences were observed under growth room conditions for aggressiveness between two *S. botryosum* isolates, SB-19 (collected from Saskatchewan) and SB-BAN (collected from Bangladesh field) (Kumar, 2007). SB-BAN had significantly higher aggressiveness than SB-19, most likely due to the fact that SB has been a major disease of lentil in Bangladesh. More disease samples should be collected from different disease infected areas to conduct a detailed study of these and previously collected isolates to determine if there is new variability in pathogenicity within and between populations at both locations. Moreover, it is unknown if there are other endemic *Stemphylium* species which may interact with *S. botryosum* to cause stemphylium blight. The effect of variability in pathogen aggressiveness and host

plant biology in diverse environments was pointed out by Ye et al. (2002) who stressed the importance of G × E interactions in plant breeding.

Lentil crops in Bangladesh have experienced SB epidemics for much longer than crops in Saskatchewan (Bakr and Zahid, 1987; Morrall et al., 2006) suggesting that the pathogen may have had more opportunity to co-evolve with the crop. Higher infection was observed in the reproductive stages of lentil growth; hence this growth stage is preferred for scoring disease severity. Kumar (2007) also suggested using the flowering stage for scoring SB consistently. In the experiment at PRC at Bangladesh, the local cultivars from Bangladesh flowered within 65-70 days, while the Canadian cultivars and RIL population started to flower after 90 days of sowing due to the influence of phenological adaptation as described by Materne and Siddique (2009). This may account for the higher DS scores for local susceptible checks since the onset of increasing susceptibility during the reproductive stages occurred much earlier in the growing season.

The ultimate goals of this study were to determine if there are any potential RILs with resistance to SB that can be used in future breeding programs, and to estimate how resistance to SB segregates in the inbred population. Although the large environmental effect evidenced in the line × location interaction might create a significant concern for reliable selection of SB resistance, some location specific lines with resistance to SB from this population can be incorporated in lentil breeding programs. These resistance sources also can be used to develop new resistant cultivars with multiple disease resistance for SB, ascochyta blight and anthracnose, as suggested by Tar'an et al. (2003).

4.5 Conclusions

Developing lentil cultivars with genetic resistance to SB is a practical way to mitigate the threat of SB under field conditions. As shown here, this can be done by transferring resistance genes from resistant germplasm or breeding lines. The current study is the first SB resistance screening study with RILs developed from parents with similar adaptation status which is important for reducing phenotypic error as suggested by Kumar (2007). A few RILs from LR-39 with significantly lower disease

severity than the resistant check ‘Eston’ in the Bangladesh environment could be used in future breeding programs to develop SB resistant cultivars for particular environments. As this study was conducted during a single season at each location, additional screening of LR-39 RILs in more environments and years could provide sufficient data for an improved estimation of the inheritance of resistance and the influence of environment.

4.6 Prologue to Chapter 5

Screening of wild germplasm accessions (Chapter 3) and the inheritance study from intraspecific populations (Chapter 4) stimulated the idea to study an interspecific population. An interspecific RIL population named LR-26 was previously developed from a cross between *L. culinaris* (‘Eston’) and *L. ervoides* (IG 72815) and was selected for screening for resistance to SB in two different environments. ‘Eston’ is common parent also used to develop the LR-39 RILs. Parent IG 72815 was found to have resistance to SB as shown in Chapter 3. This provided an opportunity to study the inheritance of resistance in segregating populations developed from a resistant × resistant interspecific cross. Results of these investigations are presented in Chapter 5.

CHAPTER 5

SCREENING OF INTERSPECIFIC RECOMBINANT INBRED LINES OF LENTIL FOR RESISTANCE TO STEMPHYLIUM BLIGHT

5.1 Introduction and objectives

Results described in the previous two chapters showed that sources of resistance to SB are limited in cultivated lentil germplasm. In major production areas like western Canada, no fungicides have been registered for control of SB (Dokken-Bouchard, 2010; S. Banniza, personal communication; Kumar, 2007). Good resistance sources in the cultivated lentil species may be limiting due to the narrow genetic base of cultivated *Lens* species used in lentil breeding programs, or may be ineffective due to high selection pressure caused by rapidly evolving new pathotypes, or poor agronomic management practices. Experiments in Chapter 3 showed that wild *Lens* species are a rich resource for resistance to SB. The use of wild relatives as an important source of resistance to pest and diseases for different globally important crops including lentil were recently reviewed by Hajjar and Hodgkin (2007). The use of wild *Lens* species to develop intra- and interspecific hybrids followed by generation advance through embryo rescue, grafting, cutting or tissue culture technique have been reported (Cohen et al., 1984; Fiala et al., 2009; Vail et al., 2012). The development of the F_1-population of one interspecific cross between *L. culinaris* and *L. ervoides* was also based on a modified crossing technique followed by *in situ* ovule rescue technology (A. Tullu, Dpt. Of Plant Sciences, University of Saskatchewan, personal communication).

Our previous SB screening study with *Lens culinaris* genotypes and accessions from all six wild species revealed there are numerous sources of resistance for multiple diseases (Tullu et al., 2006; 2010; Chapter 3). Wild lentil accessions in the previous studies were initially selected on the basis of having resistance to ascochyta blight caused by *Ascochyta lentis* Vassiljevsky and anthracnose caused by *Colletotrichum truncatum* (Schwein.) Andrus & Moore (Tullu et al., 2006; 2010). More than 80% of the accessions from *L. ervoides, L. nigricans* and *L. lamottei* which belong to the secondary gene pool (Cubero et al., 2009) had resistance to SB, and more than 60% of the accessions of the remaining three wild *Lens* species were resistant to SB. The *L. ervoides* accession IG 72815 had a resistant disease reaction to SB as well as

resistance to anthracnose and ascochyta blight. The Canadian cultivar 'Eston' was found to be resistant to SB. Both genotypes were previously used to develop an interspecific recombinant inbred line (RIL) population named LR-26 (A. Tullu, Dept. of Plant Sciences, University of Saskatchewan, personal communication).

Three intraspecific RILs have been phenotyped for SB disease reactions, with the conclusion that inheritance of resistance was quantitative (Kumar, 2007; Saha et al., 2010; Chapter 4). A genetic map constructed by Saha et al. (2010) based on an intraspecific cross between two *Lens culinaris* parents showed quantitative inheritance of resistance to SB. Interspecific lentil populations were previously screened for anthracnose and ascochyta blight resistance (A. Tullu, Dpt. Of Plant Sciences, University of Saskatchewan, personal communication; Vail et al., 2012; Fiala et al., 2009), but no such evaluations have been conducted for SB. Evaluation of LR-26 under greenhouse conditions revealed that resistance to race 1 and race 0 of *Colletotrichum truncatum* was polygenic (A. Tullu, Dept. of Plant Sciences, University of Saskatchewan, personal communication).

This study was conducted with the objectives of phenotyping the interspecific LR-26 RILs in both field and indoor conditions, and of determining the pattern of inheritance of resistance to SB. It was hypothesized that this RIL population could provide evidence for pyramided genes for resistance to SB derived from both resistant parents. This information could be used to improve breeding strategies for developing durable SB resistance in lentil.

5.2 Materials and methods

5.2.1 Plant materials

RILs of LR-26 were derived from the interspecific cross made in 2002 at the Crop Development Centre of the University of Saskatchewan using the procedures described by Fiala et al. (2009). Generations were advanced from F_2 to F_8 by single-seed descent. The Canadian *L. culinaris* cultivar 'Eston' is a small-seeded early maturing green lentil cultivar with yellow cotyledons. It is a "Persian type", originating from Turkey and was identified as a highly susceptible line to anthracnose and ascochyta blight (Slinkard and Bhatty, 1981; Tullu et al., 2010, Armstrong-Cho et al., 2012). The *L. ervoides* parent IG 72815 is a very small seeded accession

originating from Turkey, obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA), Syria. This accession is resistant to anthracnose, ascochyta blight and SB (Tullu et al., 2006, 2010; Chapter 3).

An array of 123 RILs from LR-26 was selected on the basis of seed availability for screening for SB reaction in two different environments. The first was a field environment at the Pulses Research Center (PRC), Bangladesh Agricultural Research Institute (BARI), Ishurdi, Bangladesh in 2011-12. The second environment was in the agricultural greenhouse of the University of Saskatchewan. The *L. culinaris* cultivars 'Eston' of the LR-26 RIL population and 'CDC Glamis' which are resistant and susceptible, respectively (Chapter 3) and the *L. ervoides* parent IG 72815 were included. Two cultivars from Bangladesh, ILL 8006 (released as BARIMasur-4) and ILL 5888 (released as BARIMasur-1 or Utfala) with known SB disease reaction were also included as resistant and susceptible checks, respectively, at both locations. BARIMasur-1 is a selection from a local land race, released in 1991 as a high yielding and less disease susceptible cultivar (Sarker et al., 2004) which became highly susceptible to SB within a few years of its release. BARIMasur-4 was released on 1996 as a SB-resistant cultivar, developed through a collaboration of BARI and ICARDA (Sarker et al., 2004). It has been widely cultivated in Bangladesh since it was released.

5.2.2 Plant establishment and inoculation of LR-26 for SB testing under field conditions at PRC, Ishurdi, Bangladesh

Similar procedures as described in section 4.2.2 were used to conduct the field experiment with LR-26 except that the RIL population was planted with three replicates instead of four in a randomized complete block design arrangement. Special precautions were taken to sow the seeds of very small-seeded lines at 2 cm depth instead of the 3 cm depth used for others.

5.2.3 Plant establishment and inoculation of LR-26 for SB testing under greenhouse conditions at the University of Saskatchewan

Six seeds of each of 127 LR-26 RILs and the same four checks mentioned above were planted in 10-cm plastic pots filled with the soil-less growth medium Sunshine Mix

#4 (Sun Grow Horticulture, Vancouver, British Columbia, Canada). The experiment was conducted in a randomized complete block design with 4 replications. Due to the large number of plants to be assessed, replicate pots per entry were prepared and seeded at five-day intervals to allow for detailed disease assessments of each replication to be completed within one day. All *L. culinaris* checks were planted one week after the seeding of LR-26 RILs to help synchronize growth stages at the pre-flowering to flowering stage for simultaneous inoculation of all entries in each replication. Pots were watered after sowing as required. After germination, plants were thinned to four per pot. A soluble mixture 20:20:20 NPK at 2 gL^{-1} water was applied once per week after emergence. Insects were controlled in the greenhouse as required using various control and repellent measures. Temperature and light were maintained as described previously for greenhouse experiments. Inoculation was also done by following the same procedure described in section 3.2.4.

5.2.4 Disease scoring and analysis

Disease severity was recorded at 120 days after sowing in the field experiment at PRC in Bangladesh, and 15 days after inoculation in the greenhouse at the University of Saskatchewan. Disease scoring, analysis and grouping of LR-26 lines in five disease resistance groups was done in the same manner described in section 4.2.3. Data from both locations were analyzed with pooled data analysis to estimate the variation due to RIL × location interaction. Pooled data analysis revealed heterogeneous variances for lines and locations (Appendix 17). When data were transformed, no reduction in heterogeneity occurred, and modeling heterogeneous variances was technically not possible. To obtain an approximate estimate of contributions of variables to the variation a simple mixed model analysis was conducted

5.3 Results

5.3.1 Disease severity of LR-26 under field conditions at PRC, BARI, Ishurdi, Bangladesh

First stemphylium blight symptoms appeared in the local susceptible check ILL 5888 and a few LR-26 RILs in mid-December 2011. Within a few days, infected plants recovered from this light infection by producing new vegetative growth with the exception of a few less vigorous lines. The main infection started in early January when foggy conditions occurred, resulting in relatively high temperature (21°C) and

humidity (86%). The cool and dry winter conditions with low precipitation in January (24 mm) and March (3.2 mm) were similar to those of previous years. However, approximately 5-6 hours per day of continuous morning fog provided sufficient moisture to keep the leaf surface wet throughout the entire cropping season, which was important for rapid disease progress. Disease infection on the unadapted checks and most of the RILs started 20-25 days after disease development on local checks. Infection proceeded rapidly after the first shower on 9th January. The average minimum (17°C) and maximum (25°C) temperatures from January to March in 2012 were favorable for disease progress (Appendix 18).

By mid-January, all rows of the local susceptible check ILL 5888, which bordered all genotypes, were highly infected with SB and provided abundant inoculum for infection of adjacent RILs and checks. The absence of any other diseases allowed accurate scoring of stemphylium blight. The analysis of variance showed significant differences among the RILs and checks with a range of DS from 5.0% (LR-26-170) to 71.7% (ILL 5888) (Appendix 14). No significant differences were observed among the two resistant parents 'Eston' (DS = 21.7%) and IG 72815 (DS = 15.0%), and the local resistant check, ILL 8006 (DS = 25.0%). The three resistant checks had significantly lower DS compared to both susceptible checks, CDC Glamis (DS = 55%) and the significantly more susceptible ILL 5888 (DS = 71.7%). The local susceptible check ILL 5888 had higher DS than all 123 RILs. None of the RILs had significantly lower DS than the resistant *L. ervoides* parent IG 72815, though 23 lines had nominally lower mean DS than IG 72815. Six LR-26 RILs had significantly lower DS than the resistant check 'Eston' and were considered very resistant. Sixty-eight RILs had similar DS compared to 'Eston'. Of the remaining 49 RILs, 40 were rated susceptible due to their statistically similar DS compared to CDC Glamis and 9 had intermediate DS scores, between those of 'Eston' and CDC Glamis. None of the lines had significantly higher DS than CDC Glamis. The frequency distribution of RILs based on their SB disease score showed a continuous distribution (Figure 5.1A)

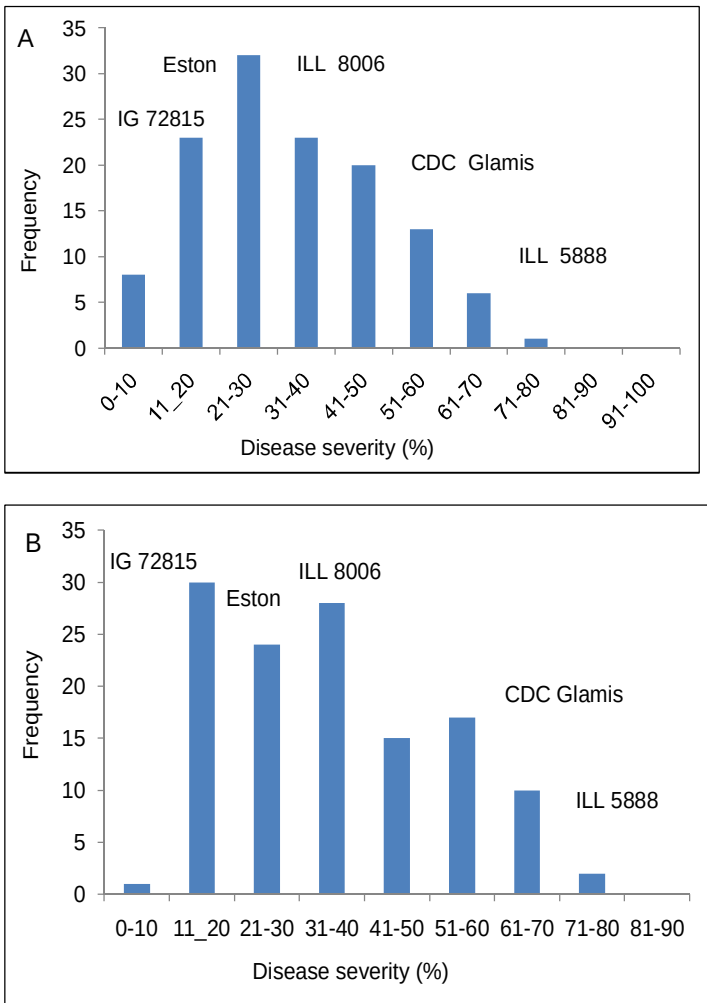

Fig. 5.1 Distribution of mean stemphylium blight severity scores for 123 RILs of the interspecific lentil population LR-26 screened at (A) Pulse Research Centre, Ishurdi, Bangladesh in 2012 and (B) in the agricultural greenhouse of the University of Saskatchewan.

5.3.2 Disease severity of LR-26 under greenhouse conditions at the University of Saskatchewan

Four days after inoculation, infection started with the appearance of tiny lesions on the susceptible *L. culinaris* checks CDC Glamis and ILL 5888. Analysis of variance revealed significant differences for DS among RILs and checks with a significant number of transgressive segregates for resistant disease reaction (Figure 5.1B; Appendix 15). The highest and lowest DS was observed on ILL 5888 (77.5%) and LR-26-66 (9.1%), respectively. The *Lens ervoides* parent IG 72815 (18.5%) was more

resistant to stemphylium blight than 'Eston' (33.1%) ($P > 0.003$), both of which had significantly less DS than the susceptible checks. Only RIL LR-26-66 had significantly lower DS than the resistant parent IG 72815 (18.5%) whereas 43 RILs had significantly lower DS than 'Eston' and were considered very resistant. RIL LR-26-294 (DS = 71.3%) was the only very susceptible line with significantly higher DS than CDC Glamis (DS = 61.9%), but not compared to ILL 5888. A total of 24 lines were susceptible with similar DS compared to CDC Glamis. Disease severity scores similar to the resistant cultivar 'Eston' were observe for 39 RILs and 16 lines were rated as intermediate.

Table 5.1. Consistency in disease reactions of *Lens culinaris* recombinant inbred lines (RILs) from the population LR-26 tested for stemphylium blight resistance in the greenhouse at Saskatoon and in the field at the Pulses Research Centre, Ishurdi, Bangladesh.

Disease Reaction	No. of LR 26 lines
VR at both locations	3
R at both locations	24
I at both locations	1
S at both locations	10
VR at Bangladesh but R at greenhouse	2
VR at Bangladesh but S at greenhouse	1
R at Bangladesh but VR at greenhouse	27
R at Bangladesh but I at greenhouse	8
R at Bangladesh but S at greenhouse	10
I at Bangladesh but VR at greenhouse	3
I at Bangladesh but R at greenhouse	2
I at Bangladesh but S at greenhouse	2
S at Bangladesh and VR at greenhouse	11
S at Bangladesh but R at greenhouse	11
S at Bangladesh but I at greenhouse	7
S at Bangladesh VR at greenhouse	1
Total	123

VR = very resistant, R= resistant, I= intermediate, S= susceptible and VS= very susceptible

5.3.3 Pooled analysis with data from both locations

Pooled data analysis revealed that the highest contribution (49%) to the total variability observed could be attributed to the RIL × location interaction compare to RIL with 29%. Among the 123 RILs, both consistent and inconsistent disease

reactions at the two locations were observed for all of the five disease classes (Table 5.1). Lines that were rated VR to R in both locations are compiled in appendix 19.

5.4 Discussion

A high level of phenotypic variation in SB resistance was observed among RILs of the large interspecific population LR-26 after evaluation under both field and greenhouse conditions. Disease reaction among RILs at both locations showed clear segregation for resistance and susceptibility indicating interspecific transfer of different resistance genes from the two resistant parents into the hybrid lines. The range of DS was greater under greenhouse compared to field conditions in Bangladesh, and mean DS in the greenhouse was somewhat higher at 36.2 ± 17.5 compared to 32.0 ± 17.6 in the field in Bangladesh. Overall higher mean DS of 38.8 ± 19.2 and 45.9 ± 15.4 were previously reported from field evaluations in Bangladesh and Saskatoon, respectively, for LR-39, the *L. culinaris* intraspecific 'Eston' (R) × PI 320937 (S) population (Chapter 4).

The resistant parents 'Eston' and IG 72815 originated from Turkey, which is part of the centre of origin of cultivated lentil (Cubero et al., 2009). A large proportion of the accessions of wild lentil species evaluated at the Crop Development Centre of the University of Saskatchewan originated from this area and showed resistance to several diseases. This could be due to the positive relationship between abundance of variability of pathogen species with diversity of resistance in host species in the center of origin or diversity (Hanounik, 1979; Cubero et al., 2009). The susceptible check ILL 5888 from Bangladesh had the highest DS both in the Bangladeshi field and in the greenhouse at Saskatoon. This genotype would be a reliable highly susceptible check for future screening of SB resistance. .

Among the RILs, about 46% of RILs were consistently rated in the very resistant to resistant range at both locations compared to 12 % of consistently susceptible RILs. Individually, 60 and 67% of RILs had VR to R disease reaction at Bangladesh and in the greenhouse experiment, respectively (Appendix 20). A higher proportion of resistant RILs in LR-26 compared to LR-39 may be due to segregation of resistance genes or alleles in the RILs from the two SB resistant parents 'Eston' and IG 72815.

However, the true number of genes or alleles conferring resistance to SB still requires clarification. A major dominant gene (*LCt2*) with some minor genes, which influenced the resistance level of intraspecific RILs to race 1 of *C. truncatum* was reported in cultivated *Lens* (Tullu et al., 2003). The remaining 42% of RILs in the current study had inconsistent disease reactions between the two locations that could be due to the differences in isolates, the influence of environmental factors and possibly the presence of more than one *Stemphylium* species causing SB.

The effects of temperature, moisture and light on sporulation, germination and disease development of SB were reviewed by Mwakutuya (2006). In the greenhouse study, temperature and light were regulated to create long day conditions with high humidity. In the field in Bangladesh, fluctuation of day and night temperatures and moisture levels could have interrupted or accelerated disease severity. A mixture of isolates is likely present in Bangladeshi lentil fields considering that this pathogen produces airborne conidia, whereas only one Canadian isolate was used for the greenhouse study. This could influence disease rating across the two environments.

Clear identification of the responsible *Stemphylium* species may be required as well. Leaf spot of asparagus (*Asparagus officinalis*) was reported to be caused by both *S. vesicarium* and *S. botryosum* in Victoria, Australia (Cunnington and Irvine, 2005). Gilbert and Parker (2010) reported significant differences in infection, necrosis development and leaf senescence in a range of native and exotic clovers (*Trifolium* and *Medicago* spp.) after screening with similar strains of *Stemphylium solani*.

No detailed population studies of *S. botryosum* have been conducted to date to determine whether physiological races exist in this species. Significant differences in aggressiveness were found between the highly aggressive isolate from Saskatchewan (SB-19) used in this study for greenhouse screening and one isolate collected from Bangladesh (SB-BAN) (Kumar, 2007). SB-BAN was more aggressive than SB-19. Differences in aggressiveness, if true for the populations of this pathogen in Canada and Bangladesh as a whole, could also influence disease reaction within RIL populations tested in different locations, and may be one reason for inconsistencies between the two experiments. Estimating the contribution of pathogen variability and host responses to local pathogen populations is important, and its impact on the G × E

interactions was discussed previously in the ascochyta blight – lentil pathosystem (Ye et al., 2002).

Due to its geographical position, seasonal fluctuations in Bangladesh are less pronounced than in Canada, which helps to forecast disease development in order to take control measures against SB at the early stages of development. However, lentil has been grown in a particular region every year which has increased the risk for inoculum build-up of certain diseases. For instance, *S. botryosum* overwinters on seed and as mycelium on plant debris (Saha, 2010), providing inoculum for the subsequent year, and indeed, SB is a consistent threat to lentil production in the region. The maximum DS of susceptible RILs from the LR-26 population, compared to local cultivars appeared 20-25 days later than that of local cultivars. Flowering of the LR-26 population was delayed compared to local cultivars under the short photoperiod of the Bangladeshi winter, and may have influenced the disease progress, as suggested by Summerfield et al. (1985). A positive relationship between post-flowering stages with SB severity was supported by observations made by both Kumar (2007) and Saha (2009).

The frequency distribution of LR-26 RILs based on DS scores from the greenhouse test was continuous, but different than the distribution in the field experiment in that there was more than one peak close to the resistant parents and checks, possibly indicating the presence of more than one major gene controlling resistance to SB-19. Saha (2010) studied inheritance of SB resistance and reported some non-allelic interactions between responsible genes conferring resistance that could influence quantitatively inherited traits as well as phenotypic expression. Quantitative inheritance of resistance was also reported by Fiala et al. (2009) in an interspecific RIL population of lentil screened for another important lentil pathogen, *Colletotrichum truncatum*.

5.5 Conclusions

Among the approaches to increase productivity of lentil, the development of cultivars with genetic resistance to SB could be an economically and environmentally sound approach. Introgression of desired resistance sources from wild *Lens* to the cultivated species appears to be a viable approach. Indeed, some RILs, such as LR-26-170, LR-

26-157, LR-26-79, LR-26-91, LR-26-98, LR-26-194, LR-26-110, LR-26-125, LR-26-132, LR-26-151 (Appendix 20) with better resistance for SB than the current cultivars, could be used in the lentil breeding program to improve resistance to SB in cultivated lentil. However, further studies with some selected resistant RILs at several locations and years could provide possible further evidence to identify the best candidates for introgression of SB resistance in the breeding program. In future, construction of a linkage map and QTL mapping, and the identification of SB resistance candidate genes from the LR-26 interspecific RILs could be the potential way to develop robust molecular markers for tracking introgression of SB resistance genes. The selected resistance sources will also facilitate the development of a new breeding strategy based on pyramiding novel genes into previously developed cultivars with resistance to other diseases.

CHAPTER 6

6. GENERAL DISCUSSSION

Canada is now the world's leading lentil producer. Saskatchewan produces more than 95% of the Canadian lentil crop and also has been the major contributor to global green and red lentil exports. Saskatchewan has potential to further increase area and production based on global trends of increased consumption of lentils. Research and development efforts are underway at the Crop Development Center (CDC) of University of Saskatchewan with the overall goals of higher yield, better quality and disease resistance. Developing cultivars with resistance to different plant pathogens requires continuous research work because of the biological dynamics of either overcoming resistance by existing pathogens or infection by newly arrived pathogens. Ascochyta blight and anthracnose of lentil are more common in temperate North American lentil growing areas, but the appearance of new diseases like stemphylium blight in Saskatchewan since 2001 (Holzgang and Pearse, 2001) has become a major concern for lentil cultivation. This concern may be increasing since the lentil production area has expanded, partly due to the availability of cultivars with resistance to ascochyta blight and anthracnose (Vandenberg and Morrall, 2002). It may be timely to intensify further investigations into identifying resistance sources for SB from the *Lens* gene pool and to develop a clear understanding of the underlying genetics to speed up the development of improved resistant cultivars.

The current project goals were to screen resistance sources for SB from different species of the *Lens* genus and to estimate the inheritance of resistance from parents to their intra- and interspecific RILs. The overall hypothesis of this study was that accessions from both cultivated and wild species have resistant sources for SB which could be transferable from parent to their intra- and interspecific RILs developed by resistant (‘Eston’) × susceptible (PI 320937) and resistant (‘Eston’) × resistant (IG 72815) parents, respectively. Chapter 3 of this dissertation was compiled with the materials and techniques used to screen all the seven taxa of the genus *Lens* individually and results from each of the separate experiments. The results from studies investigating the inheritance patterns of intra- and interspecific RILs, each of them evaluated in two different locations, were described in chapter 4 and 5, respectively.

Overall, very few resistant genotypes were found from cultivated species which was also reported in two previous studies (Kumar, 2007; Saha, 2009). This low variability for resistance is really an important bottleneck for developing resistant cultivars using cultivated species. Few unadapted cultivated *Lens* accessions were found with resistance to SB. Those that were resistant, such as ILL 8006 and ILL 1704, are adapted to the short day Asian or Mediterranean regions but not in temperate areas. These could be given priority to introgress resistance into improved cultivars though it would be necessary to overcome day length sensitivity interactions. More accessions from the wider gene pool of cultivated species can be evaluated to obtain additional reliable sources of resistance. For example, ICARDA has a large collection of cultivated species from all over the world and evidence exists that resistance can be identified in these collections. It is, however, a concern that marker analysis showed very low genetic variability among *L. culinaris* accessions from the ICARDA, USDA and Australian gene banks (Tullu et al., 2010). This narrow range of variability and development of more virulent biotypes of pathogens increases the importance of identification and incorporation of potential resistance genes from wild species (Rao et al., 2003)

Introgression of resistance genes from wild relatives, especially for disease resistance, was reported in several crops, such as sorghum (*Sorghum bicolor* (L.) Moench), pearl millet (*Pennisetum glaucum* R. Br.), chickpea (*Cicer arietinum* L.), pigeon pea (*Cajanus cajan* (L.) Millsp.), groundnut (*Arachis hypogaea* L.) (Rao et al., 2003), potato (*Solanum tuberosum* L.) (Vleeshouwers et al. 2011) and wheat (Polak and Bartos, 2002). Two Canadian lentil varieties, CDC Robin and CDC Redberry developed through hybridization within the cultivated species, were reported to have improved resistance for ascochyta blight and to some isolates of *C. truncatum* (Vandenberg et al., 2002b; 2006). CDC Robin was developed by crossing between a *L. culinaris* parent and a breeding line "ESOR-3-6-1" with *L. orientalis* background (Vandenberg, unpublished).

The encouraging results from wild species screening of this study revealed remarkably high frequency of sources of resistance to SB. A hybridization barrier between different species, in some cases, however, remains an obstacle for

introgression of desirable genes or alleles from wild relatives to cultivated lentil. These crossability barriers were the basis for separation of the species of *Lens* genus into different gene pools (Cubero et al., 2009). Fratini et al., (2004) grouped five *Lens* species into three different gene pools on the basis of crossability, with L. *culinaris* and *L. tomentosus* in the primary, *L. odemensis* and *L. nigricans* in the secondary and *L. ervoides* in the tertiary gene pool. Some recently developed protocols that help overcome these barriers have provided an opportunity to enhance introgression of resistance from wild species to cultivated lentil (Vail et al., 2012; Fiala et al., 2009). Recently another modified protocol was reported to be successful to develop intra and interspecific hybrids by using accessions from all species of the genus *Lens* (S. Saha, CDC, University of Saskatchewan, personal communication).

Intraspecific hybrid populations within the wild species might help with allelism testing in the wild background (Vail, 2010). Another concern about introgression of resistance genes from wild accessions is that transfer of undesirable genes with deleterious effects may occur. Backcrossing may eliminate this linkage drag from the newly developed improved resistant breeding lines. Different techniques have already been successfully developed to rapidly advance generation time and screening techniques, for example the use of grafting and cuttings, followed by screening in tissue culture.

The two Canadian *L. culinaris* cultivars, 'Eston' and 'CDC Glamis', were used as resistant and susceptible checks in evaluations of SB resistance in the wild species. It was clearly demonstrated that a high frequency of resistance to SB occurs in most of the wild lentil species, mostly ranging from very resistant to intermediate resistance. The range of DS for *Lens orientalis* most closely resembled that of the cultivated species. However, this research was the first screening of wild species for SB resistance, and priority was given to wild lentil accessions for which previous studies had identified resistance to other diseases. The entire world collection of 500-600 accessions of wild species would need to be screened to determine the true frequency of SB resistance for each species. The current distribution of species within the collection indicates that *Lens tomentosus* and *Lens lamottei* may be under-represented. Further expansion of the wild lentil collection is a priority of recent efforts by the Global Crop Diversity Trust to preserve wild relatives of crop species,

including lentil (Global Crop Diversity Trust, 2010). The results from this research indicate that this could be of great benefit, especially in light of the rapid expansion of stemphylium blight as a major crop disease in areas like the lower Indo-Gangetic plain (A. Sarker, personal communication) .

Among the wild species, the highest number of accessions was evaluated for *L. nigricans* followed by *L. orientalis* and *L. ervoides,* a reflection of the distribution frequency of wild species within the collection. The highest percentages (100%) of SB resistant lines were found in *L. lamottei,* which has a narrow natural range in the southern part of Spain near Gibraltar. In comparison to the other wild species, *L. nigricans* and *L. ervoides* are distributed in a much wider range of environments including northern Africa, temperate Asia and Europe (GRIN, 2012). The range of genetic variability in both host and pathogen could be greater in these two species. Tullu et al. (2010) also reported the highest (58-67%) proprotion of resistant accessions from these two species for *Ascochyta lentis* in comparison to the other wild lentil species.

The inoculation procedure in some experiments used mycelial suspensions, unlike inoculation for other diseases of lentil, such as ascochyta blight and anthracnose. Though it is proven to be an effective technique for screening, a standard protocol for screening using spore suspensions could be developed to save time and cost. Research work is underway to develop a standard protocol at CDC of University of Saskatchewan.

Stemphylium blight of lentil mainly attacks the plants from the flowering stage and onwards. Planting date was manipulated for cultivated and wild species to synchronize the flowering stage to obtain more reliable data and reduce experimental error in indoor experiments. It was assumed that phenological stage was constant for the wild species, but some differences did occur. Future studies could possibly group the accessions within lentil species by phenological stages to improve inoculation and disease scoring. Very few data have been published for this type of characterization. In both field and growth room environments, *L. nigricans* appears to primarily flower under short day conditions (A. Vandenberg, pers. com.), so accessions from this species could be screened out in the Bangladeshi environment where lentil are grown

in winter under short day conditions. The other species appeared to have long day flowering responses more similar to the cultivated lentil.

Phenotyping was done in four different environments for *L. culinaris* screening which also provided the protocol for screening SB in both indoor and outdoor conditions. This protocol can help to screen more germplasm from both cultivated and wild *Lens* species in diverse environments and to identify germplasms with consistent disease reaction. Large scale experiments for marker assisted selection of resistance genes or alleles to SB are likely more reliably conducted under field conditions where more replicates can be used for evaluation purposes. Small plots that develop a desired canopy may improve calculating of DS ratings. The lentil growing areas in Bangladesh have highly reliable conditions for SB screening considering the abundance of natural inoculum. At Saskatoon, artificial environments can be created by developing specific disease nurseries with spreader plants or plots, misting systems, or storing plant debris from infected field for inoculation to encourage disease progress to allow screening.

Severe infection by SB during the pod setting stage can cause more yield loss compared to infection that starts closer to maturity. In chickpea (*Cicer arietinum*) ascochyta blight (*Ascochyta rabiei* (pass) Lab.) epidemics were observed to be more severe at the flowering and pod formation stage (Sharma et al., 2010). Vail (2010) also reported higher susceptibility to *Colletotrichum truncatum* race 0 in the partially resistant cultivar 'CDC Redberry', during the early pod formation stage. Infection by SB in the early podding stages was also observed under highly conducive environments under field conditions in Bangladesh in this study. Early infection due to SB can reduce the plant population followed by yield loss (Sinha and Singh, 1993).

Management of SB can integrate the use of resistant cultivars, timely seeding and application of foliar fungicides (Chen et al., 2009). Practice of crop rotation and use of disease free seed were also suggested as ways to reduce disease infection by SB (Wunsch, 2012). In Canada, no fungicides have been recommended yet, but pyraclostrobin was reported to be effective in reducing SB (Banniza, 2011). Changing the sowing date could allow escape from SB infection in some environments, as reported by Huq and Khan (2007). Lentil crops planted 20 days early than normal

(within 1st and 2nd week of November) had significantly less percent disease index. These results differed from earlier studies that suggested delayed seeding reduced SB severity (Bakr and Ahmed, 1992). The contrasting results may reflect changes in environmental condition in the last few decades. In Saskatchewan sowing date studies could be done to determine if the crop can escape the cool and humid condition at the later stages of crop development, which are conducive for rapid development of foliar diseases. However, the very short growing season and a high degree of variability in the continental summer climate may make this difficult to achieve.

The consistent disease reaction from both the *L culinaris* genotypes, 'Eston' and PI 320937 at four different locations allowed identification of LR-39 RILs for future investigations into the inheritance of SB resistance. Both the parents are adaptive to Saskatchewan environment but not in Bangladesh. The differences in latitude and photoperiod between these two locations have an influence on phenological expression of both parents under field conditions in Bangladesh. A similar effect was also observed in RILs developed from these two parents. But the presence of high humidity and temperature even during the later developmental stage of lentil combined with the abundance of natural inoculum from the highly infected susceptible check (ILL 5888) overcame the biological barrier for screening the population at Bangladesh field condition, similar to the results of Kumar (2007). ICARDA also has been conducting SB screening nurseries in Bangladesh with exotic germplasm from different regions of the world and has developed breeding lines and SB resistant varieties for many years (Sarker et al., 1999). However, to synchronize maturity with local check and non-adaptive genotypes or RILs, delayed sowing of local checks could be effective. Field experiments at Saskatoon under low plastic tunnels were also effective for maintaining humidity and temperature for SB progress. More spreader plants as well as plant debris could be used to increase disease pressure.

The distribution of LR-39 RILs in both environments showed quantitatively inherited resistance. Both qualitative and quantitative inheritance of SB resistance was previously reported in different crops. For example, qualitative inheritance of resistance was reported in tomato for *S. solani* whereas quantitative inheritance was observed in cotton (Behare et al., 1991; Mehta and Arias, 2001). A large number of

RILs in this study with resistant and intermediate disease reaction indicated the contribution of both major and minor genes conferring resistance. Further investigations in different locations could be helpful to refine estimates segregation of resistance in hybrid populations.

The mode of inheritance for the interspecific RILs of the LR-26 population also revealed the involvement of more than one gene, major or minor, for resistance to SB. The resistant *L. ervoides* accession IG 72815 was used as a pollen parent and the *L. culinaris* accession 'Eston' as female. The same population was used to investigate the genetics of resistance to the highly aggressive race 0 of *C. truncatum* (A. Tullu, Dpt. of Plant Sciences, University of Saskatchewan, personal communication). Two recessive genes were identified for resistance to anthracnose race 0 from IG 72815. In the SB study, the distribution of the LR-26 populations showed large numbers of lines to be in very resistant to resistant group, with a significant number of lines transgressively segregating for SB resistance. This may be due to introgression of different alleles or genes from both resistant parents in the hybrids. The parent 'Eston' was a common parent in both LR-39 and LR-26 RILs that were evaluated following the same procedures here. The development and refinement of linkage maps of these two populations could provide information about or resistance genes or alleles from the parent 'Eston'. Another *Lens ervoides* accession L01-827A was used to develop RILs of LR-59 to study anthracnose resistance (Fiala et al., 2009; Vail, 2010). This population could also be screened for SB following the protocols described in this study and additional information on SB resistance genes could be obtained.

All of the three parents 'Eston', PI 320937 and IG 72815 used to develop both LR-39 and LR-26 were evaluated in different environments. IG 72815 did not flower in the field in Bangladesh. All the RILS from LR-39 were fertile under field conditions of Saskatoon and Bangladesh. Fertility data for the LR-26 RILs was not recorded in Bangladesh, but research at the CDC has revealed high or partial sterility in many lines while they studied this population for anthracnose resistance (A. Tullu, Dpt. of Plant Sciences, University of Saskatchewan, personal communication). Fiala et al., (2009) reported sterility in LR-59 and also explained the possibility of linkage between resistance genes with genetic factors influencing sterility. These are not yet clearly understood.

To date, no study has reported QTLs for SB resistance in RILs except in the *L. culinaris* intraspecific RILs of Saha, et al. (2010). The phenotypic data of LR-39 from this study were used for QTL analysis with 364 single nucleotide polymorphism (SNPs) markers distributed in 15 linkage groups (R. Podder, unpublished data). Field data of LR-39 from Bangladesh revealed two significant QTLs for SB resistance in linkage group 2 (accounted for 15.2% with LOD value of 4.26) and 10 (accounted for 19.9% with LOD value of 5.4). Further studies will be needed with more SNP markers to verify the results and to construct linkage maps. Tullu et al., (2003) also studied the LR-39 RILs to estimate the genetics of resistance to anthracnose by using 700 RAPD and 7 AFLP markers and designated one major resistance gene named '*LCt 2*'.

The results of this project have provided detailed information about SB resistance sources from both cultivated and wild *Lens* species. The protocol for screening wild *Lens* species in indoor and outdoor condition will be helpful for future screening. The intra- and interspecific populations screening for SB in different locations will provide insight into segregation of resistance in RIL populations and the mode of inheritance of SB resistance in lentil. The difficulties and limitations of this study highlighted in parts of the discussion will help to streamline future research on SB resistance in lentil. Finally, the results of this project indicate useful pathways for using the resistance sources and for developing a robust breeding strategy for future research leading to SB resistant lentil cultivars.

Future work

1. Selected resistant wild accessions for SB can be tested further in indoor and outdoor environments followed by utilization in the breeding program to transfer resistance into improved cultivars. The very resistant accessions can be used to transfer resistance into those cultivars which have already been improved with anthracnose and ascochyta blight resistant genes.

2. Linkage maps should be constructed by using phenotypic data from this study to identify QTLs involved in SB resistance in both LR-39 and LR-26. It will be necessary to confirm that similar markers show polymorphism in accessions from cultivated and wild species of the genus *Lens*.

3. Wild lentil germplasm should be strategically collected for evaluation of SB resistance in lentil improvement.

4. Efforts should be made to collect isolates of *S. botryosum* from Canada, Bangladesh and other newly reported SB affected areas of the world to test their aggressiveness, genetic diversity and population structure.

5. Lentil breeding materials can be reliably screened under natural condition in Bangladesh. This could be integrated into the CDC lentil breeding program through future research collaborations.

6. An artificial disease nursery for SB could be developed for large scale screening under field conditions at Saskatoon.

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APPENDICIES

Appendix 1. Levene's Test for Homogeneity and effects of genotypes on SB severity of 10 *L. culinaris* parents at 80 days after sowing under field condition at Saskatoon, Canada, 2011 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	9	1.39	0.09	0.99
Error	29	15.98		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	9	26.2	14.69	<.0001

Appendix 2. Levene's Test for Homogeneity and effects of genotypes on SB severity of 14 *L. culinaris* parents at 21 Days after inoculation under growth house condition, 2011 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	13	12.96	1.24	0.26
Error	98	15.98		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	13	89.2	27.80	<.0001

Appendix 3. Levene's Test for Homogeneity and effects of genotypes on SB severity of 14 *L. culinaris* parents at 21 days after inoculation under greenhouse conditions, 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	13	13.32	1.13	0.342
Error	98	11.78		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	13	96.9	46.23	<.0001

Appendix 4. Levene's Test for Homogeneity and effects of genotypes on SB severity of nine *L. culinaris* parents at 21 days after inoculation under field conditions at PRC, Ishurdi, Bangladesh, 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	8	34.48	2.07	0.07
Error	26	16.63		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	8	23.2	19.13	<.0001

Appendix 5. Levene's Test for Homogeneity and effects of genotypes on SB severity of ten *L. ervoides* accessions and two *L. culinaris* checks at 21 days after inoculation under growth chamber conditions at University of Saskatchewan in 2011 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	11	85.23	4.98	<.0001
Error	81	17.10		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	11	14.1	21.34	<.0001

Appendix 6. Levene's Test for Homogeneity and effects of genotypes on SB severity of twelve *L. c. ssp. orientalis* and two *L. tomentosus* accessions with two *L. culinaris* checks at 21 days after inoculation under growth chamber conditions at University of Saskatchewan in 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	15	282.2	16.21	<.0001
Error	112	17.40		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	15	48	44.12	<.0001

Appendix 7. Levene's Test for Homogeneity and effects of genotypes on SB severity of nine *L. odemensis* accessions and two *L. culinaris* checks at 15 days after inoculation under greenhouse conditions at University of Saskatchewan in 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	10	42.64	4.03	0.002
Error	77	10.58		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	10	33	17.34	<.0001

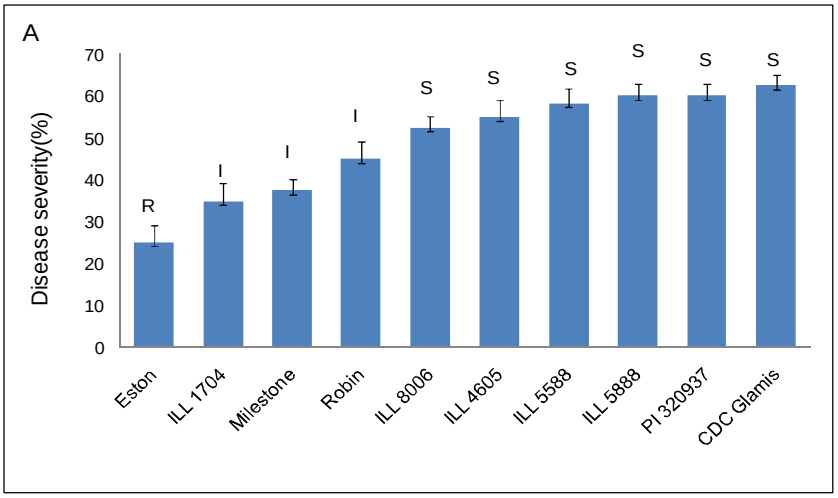
Appendix 8. Levene's Test for Homogeneity and effects of genotypes on SB severity of 18 *L. nigricans* accessions and two *L. culinaris* checks at 15 days after inoculation at greenhouse of University of Saskatchewan in 2012 based on mixed model analysis.

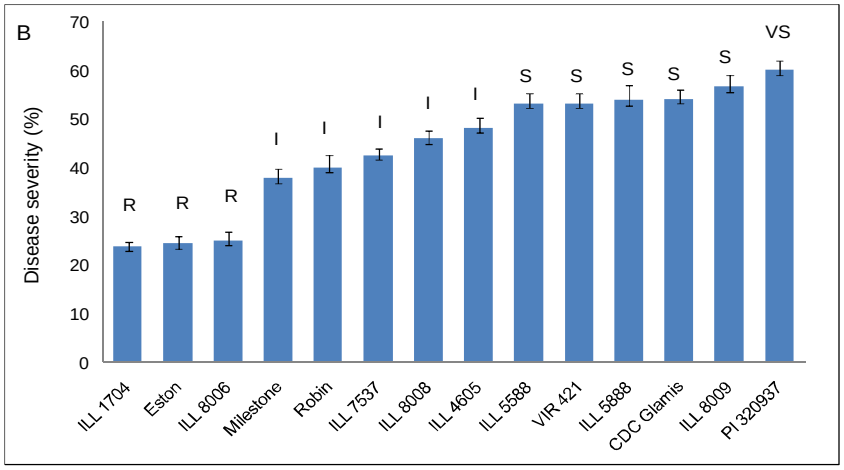
Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	19	53.32	2.65	0.006
Error	133	20.15		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	19	55.1	18.06	<.0001

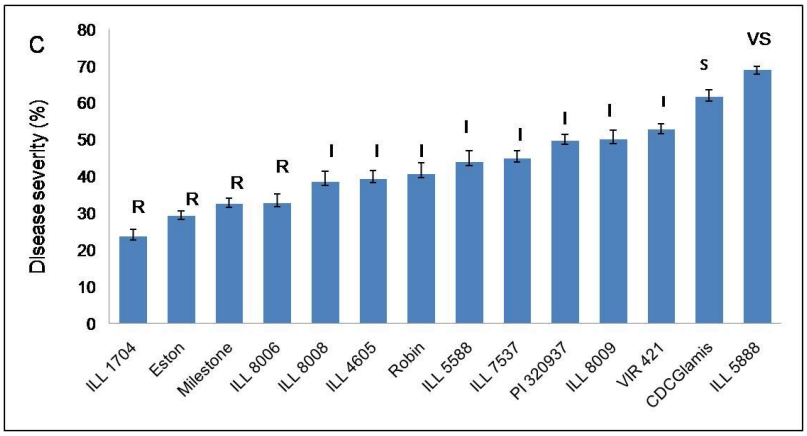
Appendix 9. Levene's Test for Homogeneity and effects of genotypes on SB severity of five *L. lamottei* accessions and two *L. culinaris* checks at 15 days after inoculation at greenhouse of University of Saskatchewan in 2012 based on mixed model analysis.

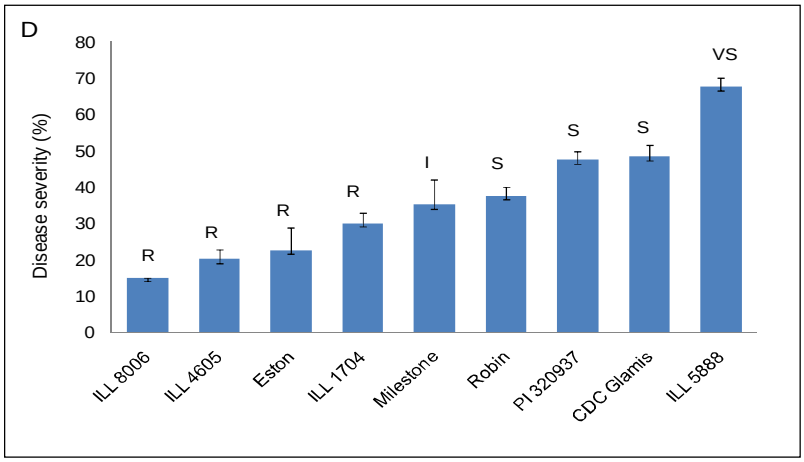
Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	6	24.14	1.84	0.1120
Error	48	13.15		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	6	6.74	47.37	<.0001

Appendix 10. Disease reaction of *L. culinaris* accessions inoculated with the isolate of *S. botryosum* (SB-19) from three different locations. A – in the field of University of Saskatchewan in 2011, B – in the growth chambers in 2011, C – in the greenhouse of University of Saskatchewan in 2012, D - in the field at Pulses Research Centre, Ishurdi, Bangladesh in 2012 inoculated with SB infected plant debris. Disease reaction: (R) - similar to 'Eston'; (S) - similar to CDC Glamis; (VS) - more susceptible than CDC Glamis; (I) - intermediate between 'Eston' and CDC Glamis. Y-bar is the standard error of the mean.

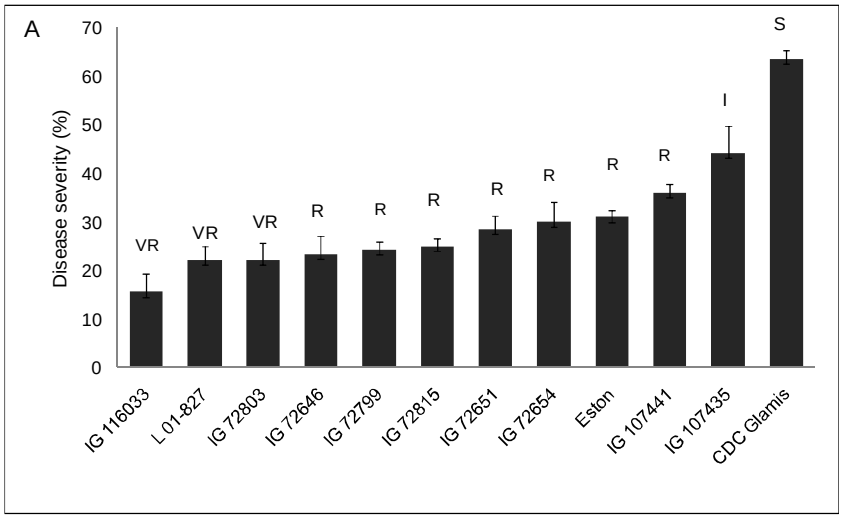


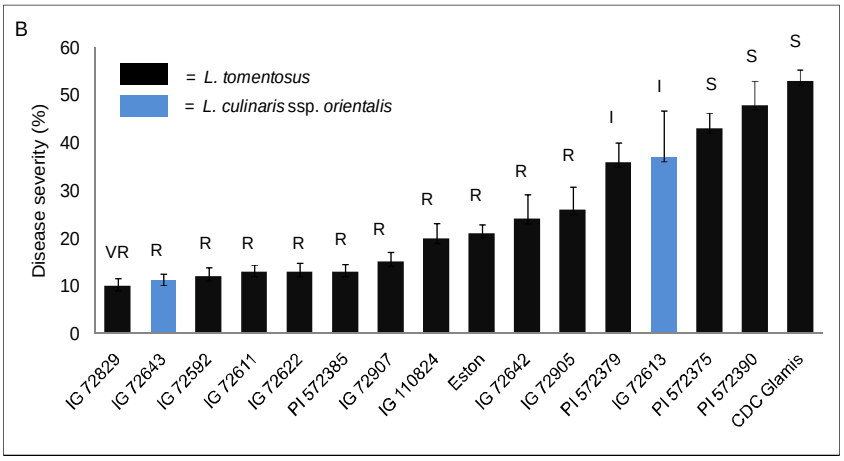


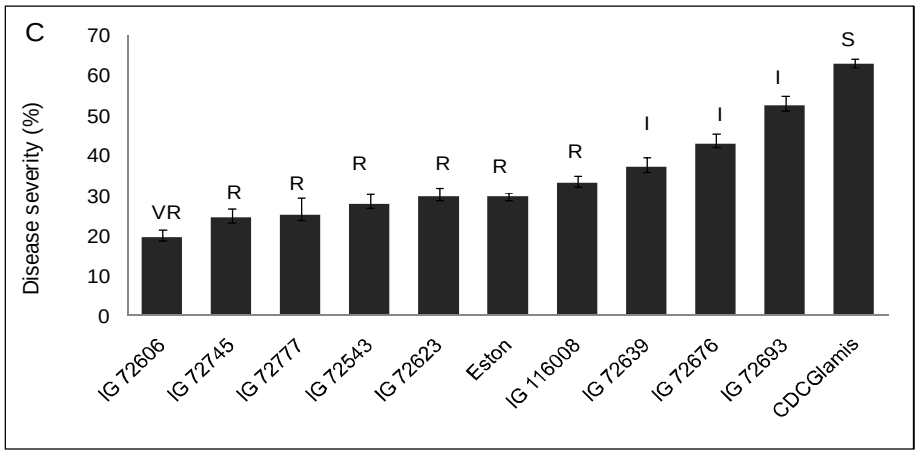

C
Disease severity (%)
80
70
60
50
40
30
20
10
0
R
R
R
R
I
I
I
I
I
I
I
S
VS
ILL 1704
Eston
Milestone
ILL 8006
ILL 8008
ILL 4605
Robin
ILL 5588
ILL 7537
PI 320937
ILL 8009
VIR 421
CDCGlamis
ILL 5888

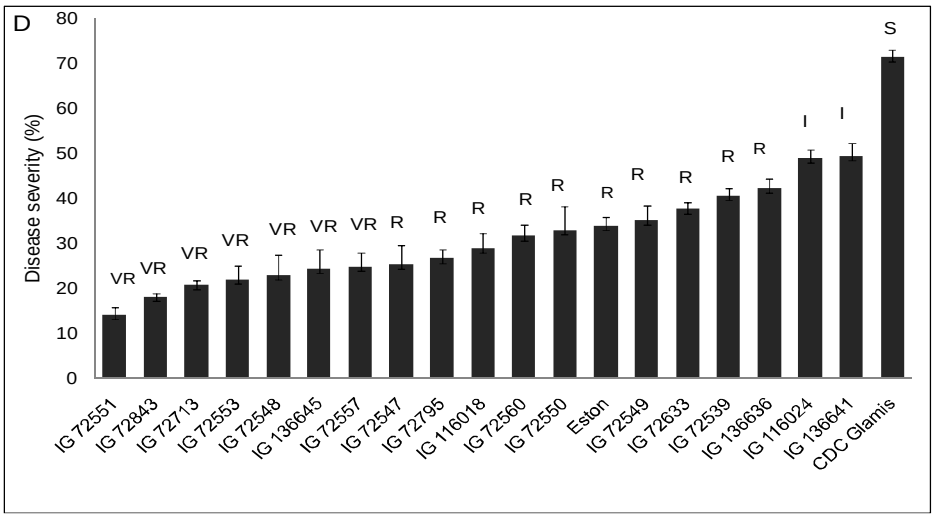

D
Disease severity (%)
80
70
60
50
40
30
20
10
0
R
R
R
R
I
S
S
S
VS
ILL 8006
ILL 4605
Eston
ILL 1704
Milestone
Robin
PI 320937
CDC Glamis
ILL 5888

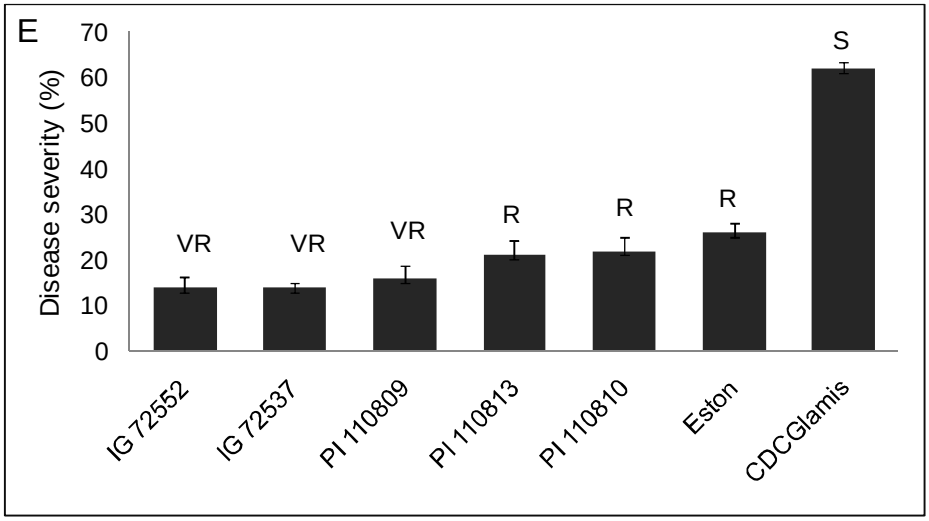
Appendix 11. Disease reaction and mean disease severity (%) of accessions from six wild *Lens* species inoculated with the isolate of *S. botryosum* SB-19. A - *L. ervoides* in growth chambers in 2011; B - *L. c.* ssp. *orientalis* and *L. tomentosus* in growth chambers in 2011; C, D & E - *L. odemensis, L. nigricans* and *L. lamottei*, respectively in greenhouse in 2012. Disease reaction: (VR) - more resistant than 'Eston; (R) - similar to 'Eston'; (S) - similar to CDC Glamis; (VS) - more susceptible than CDC Glamis; (I) - intermediate between 'Eston' and CDC Glamis. Y-bar is the standard errors of the means.






C
Disease severity (%)
70
60
50
40
30
20
10
0
VR
R
R
R
R
R
R
I
I
I
S
IG 72606
IG 72745
IG 72777
IG 72543
IG 72623
Eston
IG 116008
IG 72639
IG 72676
IG 72693
CDCGlamis


D
Disease severity (%)
80
70
60
50
40
30
20
10
0
VR
VR
VR
VR
VR
VR
VR
R
R
R
R
R
R
R
R
R
R
I
I
S
IG 72551
IG 72843
IG 72713
IG 72553
IG 72548
IG 136645
IG 72557
IG 72547
IG 72795
IG 116018
IG 72560
IG 72550
Eston
IG 72549
IG 72633
IG 72539
IG 136636
IG 116024
IG 136641
CDC Glamis


E
Disease severity (%)
70
60
50
40
30
20
10
0
VR
VR
VR
R
R
R
S
IG 72552
IG 72537
PI 110809
PI 110813
PI 110810
Eston
CDCGlamis

Appendix 12. Levene's Test for Homogeneity and effects of genotypes on SB severity of 99 lines from LR-39 including checks at 80 days after sowing under field conditions at University of Saskatchewan in 2011 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	Df	Mean square	F-Value	P-value
Genotype	99	50.34	1.88	<.0001
Error	287	26.77		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	99	284	4.65	<.0001

Appendix 13. Levene's Test for Homogeneity and effects of genotypes on SB severity of 99 lines from LR-39 including checks at 120 days under field conditions at PRC, Ishurdi, Bangladesh in 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	df	Mean square	F-Value	P-value
Genotype	99	93.75	1.39	0.018
Error	297	67.33		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	99	294	2.68	<.0001

Appendix 14. Levene's Test for Homogeneity and effects of genotypes on SB severity of 127 lines from LR - 26 including checks at 120 days after sowing under field conditions at PRC, Ishurdi, Bangladesh in 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	Df	Mean square	F-Value	P-value
Genotype	126	28.58	1.52	0.0034
Error	236	18.76		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	126	242	8.21	<.0001

Appendix 15. Levene's Test for Homogeneity and effects of genotypes on SB severity of 127 lines from LR-26 including checks at 15 days after inoculation under greenhouse conditions at University of Saskatchewan in 2012 based on mixed model analysis.

Levene's Test for Homogeneity				
Source	Df	Mean square	F-Value	P-value
Genotype	126	10.19	1.25	0.054
Error	380	8.13		
Effect	Numerator DF	Denominator DF	F-Value	P-value
Genotype	126	377	31.22	<.0001

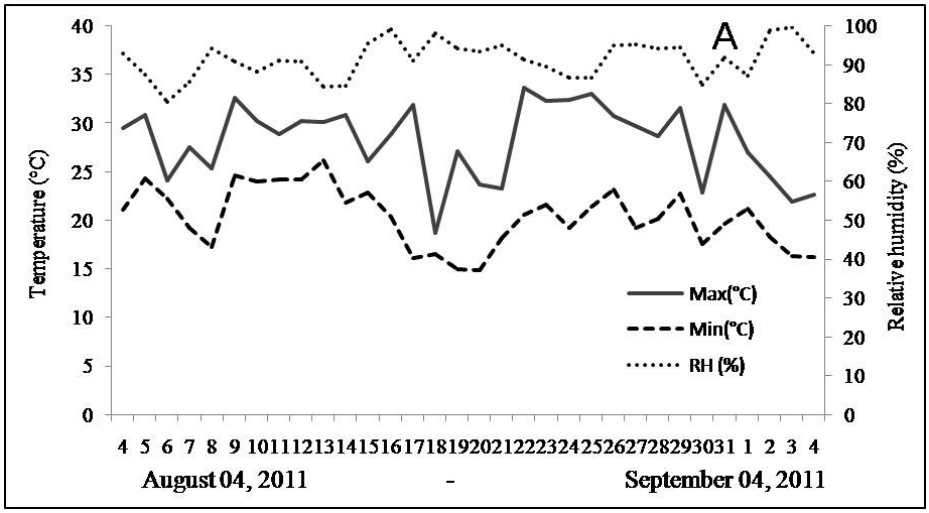
Appendix 16. Pooled analysis for estimating line × location interactions for DS of 99 lines of the LR-39 population including checks evaluated under field conditions at Saskatoon and Ishurdi, Bangladesh.

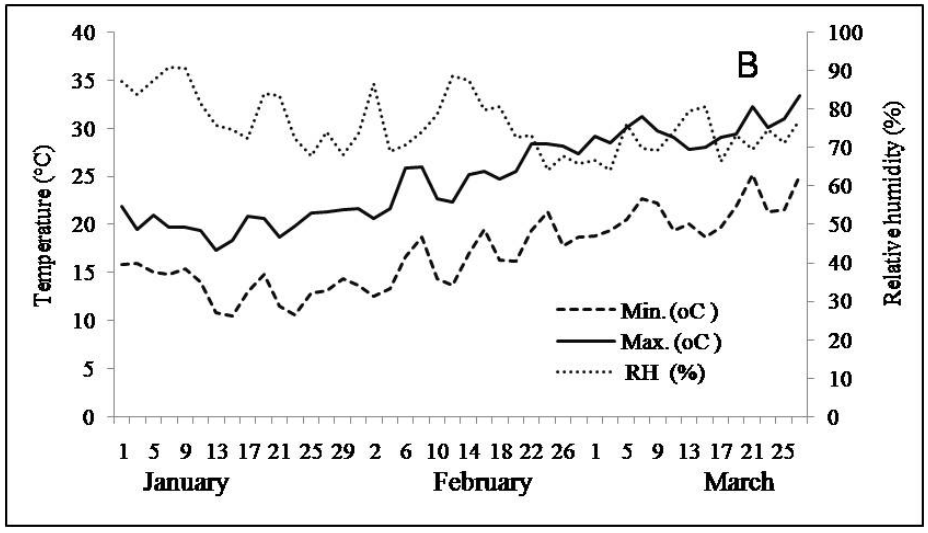
	Block (loc.)	Line	Location	Location × Line	Residual
Pooled	4.80	24.85	21.52	85.74	189.76
Bangladesh	4.22	108.54	----	----	257.34
Saskatoon	5.35	113.58	----	----	119.85

Appendix 17. Pooled analysis for estimating line × location interactions for DS of 127 lines of LR-26 populations including checks evaluated under field conditions at PRC, Ishurdi, Bangladesh and under greenhouse conditions at University of Saskatchewan.

	Block (loc.)	Line	Location	Location × Line	Residual
Pooled	0.59	94.1	7.73	156.63	58.14
Bangladesh	0.17	221.77			91.90
Greenhouse	0.84	272.67			36.35

Appendix 18. Maximum and minimum temperatures (°C), maximum relative humidity (%) recorded by Hobo data loggers under four poly-tunnels from August 4, 2011 to September 4, 2011 in field experiments at the University of Saskatchewan (A). Maximum temperature (°C), minimum temperature (°C), maximum relative humidity (%) for last three months of growing season (vegetative to maturity stage) in 2012 at PRC, Ishurdi, Bangladesh field condition (B).





Appendix 19. Disease severity of selected resistant (R) and very resistant (VR) lines in two locations of the LR-39 interspecific lentil population, parents and checks for Stemphylium blight infection when grown under natural field conditions at Ishurdi, Bangladesh in 2011-12 and when inoculated with *S. botryosum* isolate SB-19 infected spreader plants at University of Saskatchewan field in 2011. P-value is statistically compared with resistant check 'Eston'.

RIL	Saskatoon				Bangladesh			
	DS score (%)	SE	P-value	DR	DS score (%)	SE	P-value	DR
LR-39-14	35.00	5.7	NS	R	22.5	6.3	0.6	R
LR-39-16	31.25	2.4	NS	R	30	11.9	0.8	R
LR-39-20	46.25	1.2	NS	R	30	6.45	0.8	R
LR-39-36	48.75	4.2	NS	R	20	2.9	0.5	R
LR-39-37	42.50	6.6	NS	R	27.5	6.3	1.0	R
LR-39-40	37.50	2.0	NS	R	25	4.1	0.8	R
LR-39-47	32.50	5.9	NS	R	25	7.1	0.8	R
LR-39-48	41.25	4.7	NS	R	25	4.1	0.8	R
LR-39-51	26.25	5.1	NS	R	27.5	2.5	1.0	R
LR-39-52	30.00	4.5	NS	R	30	6.5	0.8	R
LR-39-55	25.00	4.1	NS	R	30	6.5	0.8	R
LR-39-60	37.50	7.5	NS	R	27.5	7.5	1.0	R
LR-39-64	22.50	5.2	NS	R	22.5	6.3	0.7	R
LR-39-71	43.75	5.1	NS	R	20	6.5	0.5	R
LR-39-72	45.00	3.5	NS	R	25	5.8	0.8	R
LR-39-73	16.67	1.7	*	VR	47.5	9.5	0.1	S
LR-39-74	30.00	2.9	NS	R	22.5	2.5	0.7	R
LR-39-77	46.67	4.4	NS	R	20	2.9	0.5	R
LR-39-79	43.33	8.3	NS	R	20	6.5	0.5	R
LR-39-84	28.75	5.1	NS	R	27.5	7.5	1.0	R
LR-39-86	40.00	9.1	NS	R	17.5	6.3	0.4	R
LR-39-89	56.25	5.5	NS	S	7.5	2.5	**	VR
LR-39-94	48.75	6.2	NS	R	25	7.1	0.8	R
LR-39-97	42.50	7.8	NS	R	27.5	4.8	1.0	R
LR-39-98	46.25	5.5	NS	R	27.5	11.1	1.0	R
CDC Glamis	66.25	2.3	***	S	55	***	*	S
Eston	33.75	7.9	-	R	27.5	-	-	R
ILL5888	65.00	2.9	***	S	80	***	***	VS
PI320937	55.00	5.8	*	S	55	*	*	S

DS = Disease severity in %; SE = Standard error; DR = Disease reaction; NS = non significant; * = significantly lower DS than 'Eston', *** = highly significantly lower DS than 'Eston'.

[a] = Canadian resistant check, b = Bangladeshi resistant check

c = Canadian susceptible check, d = Bangladeshi susceptible check

Appendix 20. Disease severity of selected resistant (R) and very resistant (VR) lines in two locations of the LR-26 interspecific lentil population, parents and checks for stemphylium blight infection when grown under natural field conditions at Ishurdi, Bangladesh in 2011-12 and when inoculated with *S. botryosum* isolate SB-19 in the greenhouse at University of Saskatchewan in 2012. P-value is statistically compared with resistant check 'Eston'.

	Bangladesh				Greenhouse			
RIL	DS score (%)	SE	P-value	DR	DS score (%)	SE	P-value	DR
LR-26-107	21.7	3.3	NS	R	35.6	1.6	NS	R
LR-26-110	11.7	3.3	NS	R	21.5	2.4	**	VR
LR-26-115	31.7	3.3	NS	R	39.4	2.4	NS	R
LR-26-125	11.7	3.3	NS	R	16.9	2.6	**	VR
LR-26-128	13.3	7.3	NS	R	16.9	2.8	**	VR
LR-26-13	31.7	6.7	NS	R	37.5	3.7	NS	R
LR-26-132	11.7	3.3	NS	R	17.5	3.7	**	VR
LR-26-138	15.0	5.8	NS	R	21.3	4.3	**	VR
LR-26-139	35.0	10.0	NS	R	36.3	3.8	NS	R
LR-26-145	13.3	7.3	NS	R	13.8	1.6	***	VR
LR-26-151	11.7	3.3	NS	R	27.5	2.3	NS	R
LR-26-157	6.7	4.4	**	VR	16.9	2.6	**	VR
LR-26-17	25.0	5.8	NS	R	12.5	2.3	***	VR
LR-26-170	5.0	5.0	**	VR	30.0	4.0	NS	R
LR-26-182	21.7	3.3	NS	R	21.3	3.0	**	VR
LR-26-184	15.0	5.8	NS	R	27.5	3.6	NS	R
LR-26-186	25.0	5.8	NS	R	38.1	4.1	NS	R
LR-26-187	21.7	8.8	NS	R	37.5	3.4	NS	R
LR-26-194	7.5	7.5	NS	R	17.5	2.9	**	VR
LR-26-20	18.3	6.7	NS	R	40.0	1.0	NS	R
LR-26-206	28.3	6.7	NS	R	19.4	2.4	**	VR
LR-26-207	18.3	3.3	NS	R	15.0	2.3	***	VR
LR-26-215	21.7	3.3	NS	R	34.4	2.8	NS	R
LR-26-22	35.0	5.8	NS	R	21.3	1.6	**	VR
LR-26-220	21.7	3.3	NS	R	22.5	2.0	*	VR
LR-26-224	25.0	5.8	NS	R	31.3	2.2	NS	R
LR-26-228	18.3	3.3	NS	R	16.3	2.6	***	VR
LR-26-235	21.7	8.8	NS	R	15.0	2.3	***	VR
LR-26-238	25.0	0.0	NS	R	40.6	1.9	NS	R
LR-26-253	35.0	0.0	NS	R	18.8	1.6	**	VR
LR-26-254	35.0	0.0	NS	R	20.0	1.4	**	VR
LR-26-257	15.0	5.8	NS	R	29.4	2.4	NS	R
LR-26-262	15.0	0.0	NS	R	32.5	2.9	NS	R

LR-26-266	21.7	3.3	NS	R	14.4	1.9	***	VR
LR-26-267	18.3	3.3	NS	R	41.3	3.8	NS	R
LR-26-274	7.5	7.5	NS	R	40.6	2.4	NS	R
LR-26-275	25.0	5.8	NS	R	38.8	4.6	NS	R
LR-26-288	15.0	5.8	NS	R	25.2	1.6	NS	R
LR-26-297	15.0	5.8	NS	R	37.5	1.8	NS	R
LR-26-32	21.7	3.3	NS	R	21.3	2.6	**	VR
LR-26-36	25.0	5.8	NS	R	15.6	3.3	***	VR
LR-26-41	11.7	3.3	NS	R	16.9	1.6	**	VR
LR-26-47	18.3	3.3	NS	R	24.4	1.9	*	VR
LR-26-49	31.7	6.7	NS	R	37.5	3.5	NS	R
LR-26-54	31.7	3.3	NS	R	23.8	2.4	*	VR
LR-26-55	21.7	8.8	NS	R	31.9	2.6	NS	R
LR-26-56	15.0	5.8	NS	R	29.4	2.6	NS	R
LR-26-64	25.0	5.8	NS	R	19.8	4.6	**	VR
LR-26-65	21.7	3.3	NS	R	40.0	1.8	NS	R
LR-26-77	21.7	6.7	NS	R	31.9	3.3	NS	R
LR-26-78	28.3	4.4	NS	R	15.6	1.9	***	VR
LR-26-79	6.7	4.4	**	VR	15.0	2.5	***	VR
LR-26-91	6.7	4.4	**	VR	11.3	3.0	***	VR
LR-26-98	6.7	0.0	**	VR	29.4	2.1	NS	R
IG 72815	15.0	3.3	NS	R	18.5	1.0	**	VR
Eston [a]	21.7	0.0	-	R	33.1	1.2	-	R
ILL 8006 [b]	71.7	0.0	NS	R	77.5	1.6	NS	R
CDC Glamis [c]	25.0	0.0	***	R	38.1	2.1	***	R
ILL 5888 [d]	55.0	3.3	***	S	61.9	1.0	***	S

DS = Disease severity in %; SE = Standard error; DR = Disease reaction; NS = non significant; * = significantly lower DS than 'Eston', *** = highly significantly lower DS than 'Eston'.

[a] = Canadian resistant check, b = Bangladeshi resistant check

c = Canadian susceptible check, d = Bangladeshi susceptible check